DOCTORAL THESIS

The immune system response to prolonged bouts of heavy-intensity exercise

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The immune system response to prolonged bouts of heavy-intensity exercise

A dissertation presented

by

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ABSTRACT

Background & Aims

Endurance exercise can cause immunosuppression and increase the risk of upper respiratory illness (URIs). The exercise-induced stress response exhibits effects on circulating immune cell concentrations and their functional characteristics. The purpose of this thesis was to assess the effect of exercise-induced stress on the immune system. Specifically, cellular and cytokine components were of high importance. The form of exercise employed was prolonged, heavy-intensity cycling bouts, chosen to produce large changes in cellular redistribution (ingress and egress). The immediate, stress-induced changes, and the subsequent changes during the recovery period post-exercise were analysed, following single and repeated bouts of exercise.

Methodology

Three studies were designed to examine the effect of prolonged (1.5 – 2 h), heavy-intensity (above anaerobic threshold) exercise on the immune system. Ten subjects were recruited for Studies One (mean ± SEM: age 24.2 ± 1.7 yr; height 1.82 ± 0.02 m; body mass 73.8 ± 2.0 kg; peak oxygen uptake 65.9 ± 2.3 $ml.kg^{-1}.min^{-1}$), Two (mean ± SD: age 24.0 ± 4.1 years; height 1.81 ± 0.05 m; body mass 73.3 ± 5.8 kg; peak oxygen uptake 60.7 ± 4.6 $mL.kg^{-1}.min^{-1}$), and Three (mean ± SD: age 23.2 ± 3.2 years; height 1.82 ± 0.06 m; body mass 74.8 ± 6.2 kg; peak oxygen uptake 59.0 ± 4.3 $mL.kg^{-1}.min^{-1}$). These three studies examined the effect of single and repeated bouts of prolonged, heavy-intensity cycling exercise on secretion of T helper cell-related cytokines following mitogen stimulation, and immune cell redistribution. Cytokines analysed were; IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL17-A, and cells examined included; neutrophils, lymphocytes, monocytes, γδ T cells (and their subsets, memory phenotypes, and specific receptor expression), and dendritic cells (and their phenotypes). Focus was directed to both the initial, exercise-induced effect, as well as the subsequent recovery period post-exercise (>6 h post-exercise). Analysis was performed via flow cytometry. Analysis of cytokines was completed using a cytometric

bead array following mitogen stimulation with either phytohaemagglutinin (PHA) (Study I) or phorbol 12-myristate 13-acetate (PMA) and ionomycin (Study III). Cell populations were identified and quantified with the use of monoclonal antibodies bound to fluorescent dyes. Data were analysed using both traditional statistics and magnitude-based inferences.

Results

Qualitative analysis revealed post-exercise changes in concentrations of plasma cortisol, IL-2, TNF, IL-4, IL-6, IL-10, and IL-17A compared to pre-exercise values. A Th1/Th2 shift was evident immediately post-exercise. Furthermore, for multiple cytokines including; IL-2 and TNF (Th1), IL-6 and IL-10 (Th2), and IL-17 (Th17) no meaningful (magnitude-based inference) change in concentration occurred until more than 4 hours post-exercise, highlighting the duration of exercise-induced changes in immune function. Study II demonstrated a significant decrease in the circulating concentration of γδ T cells and the δ2 subset up to 4 h post-exercise. Phenotypical differentiation revealed a significant decrease in circulating naïve and central memory δ2 cell concentrations. Additional analysis revealed preferential egress out of circulation of γδ T cells expressing $CD11a^{high}$ and $CD62L^{high}$. Finally, a significant decrease was evident in circulating total dendritic cell concentration at 1 h post-exercise, together with decreases in circulating myeloid dendritic cells and $CD14^{-/low}CD16^{+}$ dendritic cells up to 2 h post-exercise. Additionally, Study IIIA showed significant increases in stimulated cytokine concentrations (compared to pre-exercise) of Th1 cytokine; IL-2, and Th2 cytokines; IL-4 and IL-10. When adjusted for total leucocyte concentration at each time point, a significant increase from pre-exercise was observed in Th1 cytokines; IL-2, TNF, and IFN-γ, and Th2 cytokines; IL-4 and IL-10. However, there were no significant differences between the single and repeat exercise bout trials at any time point. Finally, Study IIIB revealed a significant exercise-induced increase in circulating concentrations of total lymphocytes, neutrophils, total γδ T cells, δ1 and δ2 subsets, total dendritic cells, and the plasmacytoid dendritic cells. Furthermore, among the γδ T cells, those with high expression of CD11a and CD62L were preferentially mobilised. However, no significant differences were

observed between the single and repeated exercise trials for any variable, except total circulating lymphocyte and neutrophil concentrations.

Conclusion

The results of the present thesis were hoped to be two-fold; 1) expand the knowledge base for a number of novel immune markers previous unexamined under severe, prolonged stress stimuli, and 2) provide further evidence of how repeated immune stimulation may be detrimental to healthy immune function when recovery between sessions is not sufficient. The first aim provided fairly expected results; γδ T cells were highly responsive to exercise-induced stress, as were DCs. Furthermore, cytokine production via whole blood or PBMC stimulation appeared to be enhanced following exercise bouts. The second aim however, resulted in some unexpected effects. Primarily, that repeated exercise did not result in a "carry-on" effect from the first exercise bout to the second may suggest repeated exercise with minimal recovery may not be as detrimental to cellular components of immune function as previously thought, provided there is adequate energy intake between sessions. Together, these results highlight the multifactorial nature of both; the stress response, and the immune system with its complex interactions of proteins, cells and structures functioning together to defend the host against invading pathogenic agents.

DECLARATION OF ORIGINAL WORK

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When I first set out, I was completely oblivious to the work ahead of me. Rolling into a PhD seemed the next logical step in the process, though I had given it surprisingly little thought when I took the plunge. In hindsight, I can't believe what I have accomplished.

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
APC	antigen presenting cell
AT	anaerobic threshold
ATP	adenosine triphosphate
BMI	body mass index
BP	blood pressure
CBA	cytometric bead array
CD	cluster of differentiation
$CD14^{-/low}CD16^{+}$ DC	monocyte-related dendritic cells
CL	confidence level
CM	central memory
CMV	cytomegalovirus
DALDA	daily analysis of life demands of athletes
DC	dendritic cell
df	degrees of freedom
EBV	Epstein-Barr virus
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	effector memory
EMRA	terminally differentiated effector memory
FSC	forward scatter channel
FVC	forced vital capacity
FEV_1	volume exhaled at end of first second of forced expiration
GH	growth hormone
h	hours
HDL	high-density lipoprotein

HEV	high endothelial venule
HIV	human immunodeficiency virus
HPA	hypothalamic–pituitary–adrenal
HR	heart rate
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
kg	kilograms
km	kilometres
LDL	low-density lipoprotein
LT	lactate threshold
MLSS	maximal lactate steady state
MHC	major histocompatibility complex
mDC	myeloid dendritic cells
min	minutes
mL	millilitres
µL	microliter
NA	naïve
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cell
NFOR	non-functional overreaching
NK	natural killer
OR	overreaching
OT	overtraining
PAR-Q	Physical Activity Readiness Questionnaire
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pDC	lymphoplasmacytoid dendritic cells
PHA	phytohaemagglutinin
pIgR	polymeric immunoglobulin receptor
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

POMS	profile of mood states
RCT	respiratory compensation threshold
REP	repeat trial
SD	standard deviation
SEM	standard error of the mean
sIgA	secretory immunoglobulin A
SIN	single trial
SSC	side scatter channel
TCR	T-cell receptor
tDC	total dendritic cells
TGF	transforming growth factor
Th	T-helper cell
TNF	tumour necrosis factor
UHPLC MS/MS	ultra high-performance liquid chromatography tandem mass spectrometry
URI	upper respiratory illness
URS	upper respiratory symptoms
VT_1	first ventilatory threshold
VT_2	second ventilatory threshold
$\dot{V}_E$	ventilation
$\dot{V}O_2max$	maximum oxygen consumption
$\dot{V}O_2peak$	peak oxygen consumption
VZV	varicella zoster virus
W	Watts
yr	years

1. INTRODUCTION

Heavy-intensity prolonged aerobic exercise regularly completed by endurance athletes is associated with transient changes in immune function, circulating leucocyte concentrations, and an increased risk of symptoms associated with upper respiratory illnesses (URIs) [1-5]. While many uncertainties remain, a rapid growth in the peer-reviewed publications since the mid 1980's has vastly increased the scope and understanding of the immune system's response to exercise. Originally, many studies utilised epidemiological evidence to identify an association between exercise "dose" and an increased incidence of symptoms associated with URIs. Later research, often measuring the concentration of specific leucocyte subsets in the peripheral circulation, noted an exercise-induced leucocytosis, influenced by the intensity and duration of the exercise. Additionally, studies examining particular interventions to improve immune system health and recovery from exercise, and studies charting the immunological impact of exercise stress on a larger scale have been undertaken. A great deal of knowledge has been amassed in the last thirty years. However, with all areas of research, the more knowledge that is discovered, the more one realises the dearth of knowledge still to be uncovered.

The immediate ingress of leucocytes in response to stress and/or exercise has been of great importance in exercise immunology research. However, less examination has surrounded the subsequent period following the cessation of the exercise-induced stress. This recovery period is important, as during this time changes in circulating stress hormone concentrations, leucocyte concentrations, and cellular function occur, each of these potentially causative agents in the exercise-induced increase in symptoms associated with URIs. Furthermore, the long-standing 'open-window' theoretical framework (discussed Chapter 1.3) suggests that it is during the period immediately following exercise that the immune system is most susceptible to invasion from infectious agents. Throughout this thesis, the immune system "recovery period" will be regarded as the time following the cessation of an acute stressor when cellular redistribution is occurring, until the point of where circulating cell concentration returns to resting levels.

The purpose of this research is to monitor immune system of highly trained cyclists in response to acute bouts of prolonged heavy-intensity exercise, and examine a number of important immune variables during the post-exercise recovery period.

In Chapter One, the literature review will examine the neuroendocrine and immunological response to stress and exercise with a particular focus on prolonged heavy-intensity exercise. Prolonged-intensity heavy exercise will be defined as exercise lasting one and a half hours or greater at or above the heavy-intensity exercise domain (i.e., above the anaerobic threshold). A number of cell types will be reviewed including lymphocytes and their subsets (mediating both innate and adaptive immunity), dendritic cells (DC) (immune system regulation) and neutrophils (early-onset innate immunity). Regulation of the immune system by neuroendocrine and cytokine mediators will also be discussed. Finally, the influence of repeated exercise bouts on the immune system, during activation and the recovery period will be considered. All the above will be considered in the context of the increased rate of symptoms associated with URIs during periods of heavy training and competition.

1.1 Upper Respiratory Illnesses in Endurance Athletes

Upper respiratory illnesses, often referred to as the common cold, are characterised by an inflammation of the upper respiratory tract. Symptoms presented including cough, sore throat, runny nose, head ache, nasal congestion, low grade fever, sinus pressure and sneezing. Illness generally lasts 7-10 days, with symptoms presenting 1-3 days after the exposure to the pathogen. It has been suggested that the pathogenic nature of URI is predominantly viral, with a small percentage of cases (~10%) caused by bacterial infections [6-8]. While athletes regularly present with upper respiratory symptoms (URS) [9, 10], debate still remains whether symptoms such as a 'sore throat' are always indicative of a URI, or rather airway inflammation without infection [11, 12]. This is due to the considerable cost and time required for identifying the underlying cause by a pathologist. Regardless of the underlying cause, any URS are likely to interrupt an athlete's training schedule, impeding their ability to train and compete optimally. Previous research in overtraining has demonstrated URS are associated with significant persisting fatigue and poor performance [13-17].

Endurance athletes regularly undergo significant physiological stress caused by large training volumes and high training intensities. Training may occur multiple times per day, every day of the week, resulting in repetitive stress-induced activation of the immune system [18-20]. This is important, particularly in the context of 'peaking' for competition, as increased rates of symptoms associated with URIs have been reported during periods of heavy training and competition. While many anecdotal reports circulate, suggesting symptoms associated with URIs are more common in elite athletes, very little documented evidence exists. Comparison between elite and general populations have highlighted that elite athletes experience URI symptoms at a similar rate to the general population [21-23], however differences remain in their temporal occurrence. While the general population exhibits the normal seasonal pattern, elite athlete symptoms associated with URIs appear to be much more prevalent during or around competitions [8, 24-26]. More frequent exhibition of URS appear to occur during periods of heavy training, the taper period, and following competition [8, 13, 14, 26-28].

A dose–response relationship has been observed between training volume and the incidence of symptoms associated with URI. For example, a year-long study of 530

runners found that those who trained less than 778 km per year had an average of 1.2 episodes of URI per year compared with runners who trained 778–1384 km per year who experienced two episodes, and runners who trained >1384 km who had 3.5 episodes per year [29]. In support of these findings Nieman et al. (1990) found that runners who exercised >97 km per week were twice as likely to display symptoms of URI compared with those who trained <32 km per week in the two months before a marathon. A similar incidence of symptoms associated with URI was also found in three studies involving ultramarathon runners, where the incidence of symptoms associated with URI was significantly higher in athletes following an ultramarathon event than in sedentary individuals [8, 30, 31]. A higher rate of symptoms associated with URI was also found in elite swimmers during four weeks of intense training [32], in elite hockey players during an intense 10-day training camp [5], in elite squash players during a 10-week training block [5], and in elite swimmers following a 12-week training cycle [3]. In contrast, another study failed to identify any relationship between an increased risk of contracting a URI following a marathon in a cohort of almost 1700 runners. However, these researchers found that athletes who had displayed symptoms associated with URI in the three weeks preceding the marathon had twice the chance of experiencing a second URI episode after the race compared with those athletes who had not reported URI symptoms in the three weeks before the race [33].

1.2 Overreaching, Overtraining, and Underperformance

Immune systems perturbations, risk of URI, and the effects of overtraining on performance are regularly discussed in tandem [13-17, 34-37]. The process of intensifying training is commonly used by athletes in an attempt to enhance performance. However the combination of excessive overload with inadequate recovery should be avoided. As a consequence, the athlete may experience acute feelings of fatigue and decreases in performance as a result of a single intense training session or an intense training period [38]. Under normal healthy training, the resultant acute fatigue after a training session can be alleviated by adequate rest period. This may be followed by a positive adaptation or improvement in performance; this is the basis of effective training programs. However, if the balance between appropriate training stress and adequate recovery is disrupted, an abnormal training response may occur and a state of overreaching (OR) may develop [39]. When athletes do not sufficiently respect the balance between training and recovery, non-functional overreaching (NFOR) can occur. If not managed properly, more severe overtraining (OT) can develop. Both NFOR and OT includes maladaptations and diminished competitive performance [40]. As mentioned above, previous research has highlighted an increased presentation of symptoms associated with URI during periods of heavy training and competition [8, 13, 14, 26-28]. This is when NFOR or overtraining are likely to occur. One of the hallmarks of overreaching and overtraining is underperformance [38, 40, 41]. Of course, in professional athletes underperformance can be detrimental to one's career, and can be compounded by bouts of URI. While, the increased presentation of symptoms associated with URI and NFOR/OT are not the same phenomenon, it is important to note the similar underlying causes. And while, NFOR/OT may not necessarily result in an increase in URI, and URI episodes are not always an indication of NFOR/OT, they both do occur during periods of heavy training, competition, and can result in underperformance.

1.3 The ‘J’ Curve & ‘Open Window’ Theories

The high frequency of illness that some elite athletes experience may cause major interruptions in training continuity and performance ability during competition. The relationship between exercise and infection risk can be illustrated by the ‘J’ curve [42], suggesting that individuals engaging in moderate physical activity have a decreased chance of displaying symptoms associated with URI compared with sedentary individuals [25]. However, an excessive training workload (both training volume and intensity) may increase the risk of contracting a URI [3, 21, 29, 32]. More recently, the ‘J’ curve theory was altered to a proposed ‘S’ curve relationship between training load and risk of infection [43]. This proposed modification reflected the separation of ‘high’ and ‘elite’ exercise loads when examining sick days per year in research previously published, where a decrease in infection risk is observed in elite athletes compared to highly trained athletes. It was suggested that to compete at the elite level athletes require not only a state-of-the-art physique, but an immune system able to withstand infections even during sever physiological and psychological stress.

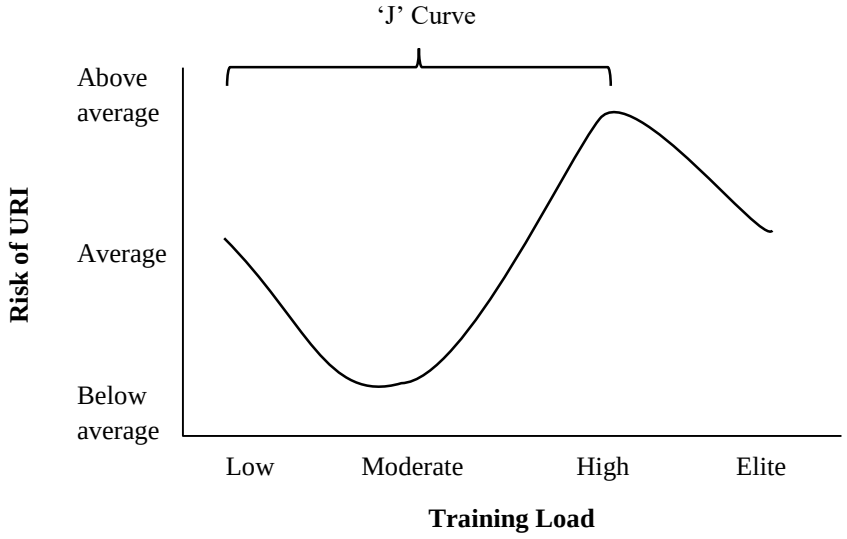

Figure 1.1. Modified version of the ‘J’ curve and ‘S’ curve of infection risk vs. physical activity [42, 43]

The ‘J’ and ‘S’ curve theories (see Figure 1.1) have been modelled to display the relationship between URI risk and exercise activity. Athletes and coaches must consider

this when planning increases in intensity and/or duration of training, as it is during these periods of increased total work when the athletes are most at risk [8, 24-26]. These occurrences of symptoms associated with URI are believed to be related to immunosuppression explained by the ‘open window’ theory as represented in Figure 1.2.

Although potentially now an out-of-date concept due to advancements in exercise and stress immunology, the ‘open window’ theory suggests that, in the hours following strenuous endurance exercise, suppression of some immune variables may cause the host to be more susceptible to pathogens that have entered the body [44]. This was hypothesised due to changes identified in lymphocyte concentrations, natural killer (NK) cell function and NK cell concentration, and secretory immunoglobulin A (sIgA) levels in the hours following exercise, possibly causing an increased risk of contracting a URI. While the concept still remains in exercise immunology, placing the ‘blame’ on a functional decrease in a single immune variable appears narrow, considering the size and relationship among the immune system’s many variables.

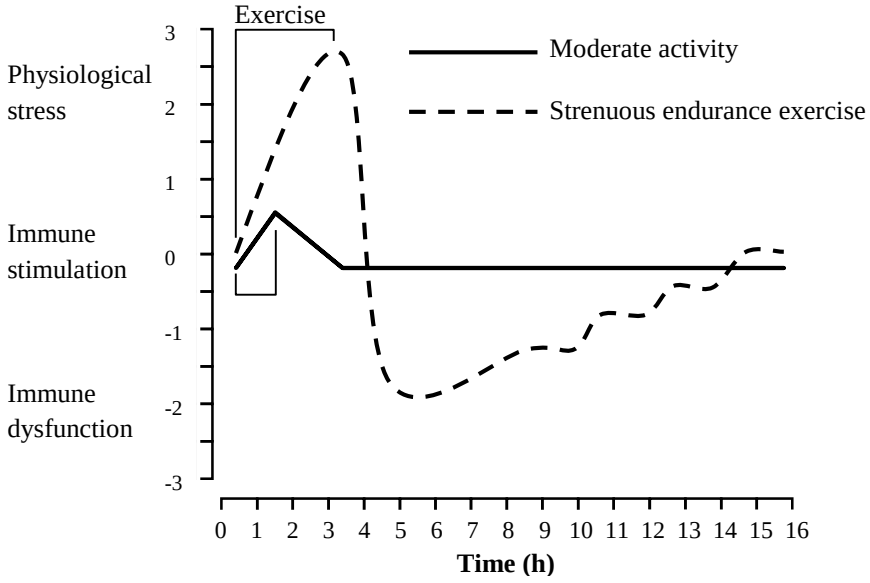

Figure 1.2. The ‘open window’ theory of immunosuppression [44]

The following sections contain a brief overview of the immune system, including changes in immune variables number and function, and regulation of these systems in response to acute bouts of endurance exercise.

1.4 The Immune System

Our environment is heavily populated by both pathogenic and non-pathogenic microbes, containing a vast array of potentially toxic or allergenic substances that threaten normal homeostasis. A huge range of pathogenic microbes replicate and spread by a diverse collection of mechanisms, threatening normal host functions [45]. To combat this threat, the immune system uses a complex array of protective mechanisms to control and usually eliminate these organisms and toxins. These protective mechanisms rely on detecting structural features of the pathogen or toxin that mark it as distinct from the host cells. Such discrimination between structures is essential to permit the host to eliminate the threat without damaging its own tissues [46]. The immune system is composed of various tissues, cells, and molecules involved in the defence of the body against infectious agents [47]. This occurs by identifying pathogens and destroying such agents [48].

1.4.1 Innate & Adaptive Immune Responses

The immune system can be divided into two sub sections: the innate (non-specific) immune response and adaptive (specific) immune response. The innate immune response is regarded as the body's "first line of defence" against pathogens and is immediately available to combat a wide range of micro-organisms, in addition to tissue damage, repair, and remodelling [45]. Because the recognition molecules used by the innate system are expressed broadly on a large number of cells, this system is able to act rapidly after an invading pathogen or toxin is encountered. Conversely, the adaptive immune system develops a specialised response to an identified pathogen, exhibiting exquisite specificity for epitopes (recognised part of antigen identified by antibodies, T cells and B cells) of individual pathogens [49].

The innate immune system is composed of physical barriers such as epithelial cell layers that express tight cell-cell contacts, the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal and genitourinary tracts, and the epithelial cilia that sweep away this mucus layer [46]. The innate response also includes soluble proteins and bioactive small molecules such as the complement proteins, defensins, and ficolins, cytokines, chemokines, lipid mediators of inflammation, reactive free radical species, and

bioactive amines and enzymes [46]. Major cells of the innate immune system include neutrophils which are “first-line” defenders against bacterial infection, dendritic cells which serve to orchestrate immune responses, macrophages that perform important phagocytic, regulatory and antigen presentation functions, and NK cells which recognize altered host cells (e.g. virally infected or transformed) [50].

In contrast to the innate immune system, the adaptive system is composed of small numbers of cells with specificity for any individual pathogen, toxin or allergen. Cells responding to a pathogen must proliferate to attain sufficient numbers to mount an effective response [47]. It is because of this mounted response that the adaptive immune system response generally expresses itself temporally after the innate response in host defence [45]. A key feature of the adaptive response is that it produces long-lived cells. These cells exist in an apparent dormant state, but can re-express effector functions rapidly after another encounter with their specific antigen [46]. Essentially, this means the adaptive response possesses the ability to manifest immunological “memory”. This immune memory allows a more effective host response against specific pathogens or toxins when they are encountered a second time [51]. It is a response that manifests exquisite specificity for its target antigens. Antigen-specific receptors of the adaptive response are encoded to allow the assembly of antigen receptors from a collection of a few hundred germ-line-encoded gene elements [46]. This permits the formation of millions of different antigen receptors, each with potentially unique specificity for a different antigen. The adaptive immune system response is mediated primarily by T and B lymphocytes and soluble proteins called immunoglobulins [50].

1.4.2 Primary & Secondary Lymphoid Tissues

Leucocytes may be found in the body distributed as single cells in the tissues and circulation, as lymphoid accumulations (e.g., Peyer’s patches), or within lymphoid organs (e.g., thymus, spleen, lymph nodes). Organs of the immune system are classified as primary or secondary lymphoid organs. Primary lymphoid organs; the thymus and bone marrow, are the site for development of lymphocytes. The secondary lymphoid organs (e.g., spleen, lymph nodes, lymphoid accumulations) trap and concentrate immunogens in

addition to providing sites where large numbers of circulating immune cells can make contact with each other [52].

The primary lymphoid organs, the thymus and bone marrow, serve as lymphocyte maturation centres. Although all lymphocytes originate within the bone marrow, those committed to the T-cell lineage migrate via the circulation to the thymic cortex. These cortical thymocytes acquire T-cell receptors (TCRs), and CD4 and CD8 surface molecules. Other lymphocytic lineage cells remain within the bone marrow, destined to become immunoglobulin-producing lymphocytes undergo their early stages of differentiation within the bone marrow to become B cells [47].

Secondary lymphoid tissues function as filtration devices removing foreign matter, dead cells, and protein aggregates from the circulation. The major secondary lymphoid organs are the spleen and lymph nodes. The tonsils and Peyer's patches also act as secondary lymphoid accumulations [47]. These organs are richly supplied with blood and lymphatic vessels facilitating movement of lymphocytes, monocytes, and dendritic cells into and out of the tissues. Specialized regions of the vasculature, called high endothelial venules (HEVs), allow movement of cells between the blood and these secondary lymphoid tissues [53, 54]. These secondary lymphoid tissues facilitate cellular interaction, providing leucocytes an environment in which they can exchange regulatory signals, undergo further development, and proliferate before re-entering the circulation [45].

1.4.3 Lymph Nodes & Lymphocyte Trafficking

The trafficking of immune cells in lymph nodes has a crucial role in immunity. Throughout the human body are hundreds of lymph nodes (~450), essential for the encounter of circulating naïve lymphocytes with antigens and antigen-presenting cells (APCs) [55]. These small round or oval-shaped secondary lymphoid organs are the site of leucocyte accumulations occurring periodically throughout the lymphatic circulatory system. They function as filters to purify lymph, the fluid and cellular content of the lymphatic circulatory system, and provide sites for mingling of lymphocytes, monocytes, and dendritic cells for initiation of immune responses [52]. Recirculation of lymphocytes through lymph nodes allows naïve lymphocytes specific for a given antigen to survey the

lymph throughout the body for the presence of their target antigen [46]. During homeostasis, lymphocytes continuously recirculate from the blood to lymphoid organs and back to the blood as often as once or twice per day [56]. This recirculation and migration (or 'homing') of lymphocytes between the blood and lymph nodes occurs via HEVs [53, 54]. Essentially, the trafficking of lymphocytes is a three step process. Initially, naïve B and T cells are transported through HEVs or afferent lymphatics via an adhesion cascade [53, 54]. Secondly, specific intranodal migration and positioning where T cells migrate to the lymph node paracortex, whereas B cells enter the B cell follicles in the cortex. Finally, egress via efferent lymphatics. If 'visiting' lymphocytes do not recognize their specific antigen, they leave the lymph node through the efferent lymphatic vessel and return to the circulation through the thoracic duct [57, 58].

1.4.4 Adhesion Molecules

Adhesion molecules are essential in leucocyte redistibution to secondary lymphoid tissues and to peripheral tissue sites of microbial invasion [47]. To maintain adequate host defence, cellular adhesion molecules and chemotactic proteins both contribute importantly to this process [51]. Three main families of cell adhesion proteins exist: selectins, integrins, and Ig domain cell adhesion molecules [46]. While mediating cell recruitment to tissues as their primary function, these molecules contribute to cell-cell interactions between leucocyte subsets and can contribute to intercellular and intracellular signalling [45]. Selectins are expressed on the surfaces of all leucocytes and on endothelial cells [46]. In addition, ligands for selectins are also present on all leucocytes. Low affinity interactions between selectin ligands on leucocytes and selectins on vascular endothelium lead to rolling of cells along the vessel wall, allowing cells to ingress into circulation and be transported [47]. Furthermore, the combined effects of integrins and Ig domain cell adhesion molecules allow leucocytes to adhere firmly to the endothelium when required [47]. Together, adhesion molecules allow for precise control of leucocytes during cellular redistribution.

1.4.5 Cluster of Differentiation

An intact immune response includes contributions from many subsets of leucocytes. The different leucocyte subsets can be discriminated morphologically by analysis of the spectrum of glycoprotein differentiation antigens that are displayed on their cell membranes [46]. These differentiation antigens are detected by their binding of specific monoclonal antibodies [47]. These cell phenotype-determining antigens are assigned cluster of differentiation (CD) numbers. There are over 350 defined CD antigens [59].

1.4.6 Major Histocompatibility Complex & Antigen Presentation

As part of normal function, the immune system is required to identify host cells that have been infected by microbes. If left to their own devices, these microbes will use the cell to multiply within the host. These infected cells that serves as a "factory" for production of progeny microbes must be identified and destroyed. A major role of the adaptive immune system is to identify and destroy infected cells [47]. Along with identification of infected cells, T cells can recognize peptide fragments of antigens displayed by APCs [45]. In order for T cells to recognise infected host cells, the immune system must differentiate "self" from "non-self". This is solved by the use of major histocompatibility complex (MHC) molecules. MHC molecules are cell surface glycoproteins that bind peptide fragments of proteins that either have been synthesized within the cell (class I MHC molecules) or that have been ingested by the cell and proteolytically processed (class II MHC molecules) [51]. MHC class I and class II molecules are similar in function: they present peptides at the cell surface to $CD8^+$ and $CD4^+$ T cells, respectively [60]. As such, these molecules are essential for specific host defence.

1.4.7 Neutrophils

Neutrophils are large granulocytes produced and matured in the bone marrow that form part of the innate immune system [47]. The process of eliminating an invading pathogen involves the phagocytosis (engulfing) of the micro-organism followed by the destruction by lysosomes and the respiratory burst, through the NADPH-oxidase system [49]. Neutrophils account for 60–70% of all circulating leucocytes [61]. Neutrophils are

recruited early to the site of infection functioning to stop an invading pathogen from replicating inside the host. This occurs via penetration of the vascular endothelium by the neutrophil, moving into the tissues at the site of infection [49]. The process of phagocytosis, by which neutrophils identify and destroy pathogens involves transduction of the neutrophil plasma membrane and cytosolic constituents, and activation of the NADPH-oxidase complex to produce reactive oxygen species targeted at the now phagocytosed pathogen to destroy it.

1.4.8 Dendritic Cells

Dendritic cells function as "professional" APCs which play a critical role in the regulation of the adaptive immune response. These cells are referred to as "professional" APCs because their principal function is capturing antigens, processing, and presenting them, and because only DCs have the ability to induce a primary immune response in naïve T lymphocytes [62]. Additionally, DCs are critical in establishing immunological memory by the maintenance of B cell function and recall responses [62]. DCs are able to activate both $CD4^+$ and $CD8^+$ T cells via antigen presentation [47]. Immature DCs originate in the bone marrow and migrate throughout the body waiting to interact with invading pathogens. These cells undergo a maturation process once they exit the circulation, into the tissue [63]. Once captured, an antigen is processed by a number of potential pathways. For MHC class I presentation to stimulate $CD8^+$ cytotoxic T cells, the antigen (or protein) is engulfed by phagocytosis or receptor mediated endocytosis into the cytosol of the DC. The antigens are further degraded in the cytosol via proteasomes and enter the endoplasmic reticulum where peptides bind to MHC class I molecules newly produced for presentation on the cell surface [52]. For MHC class II presentation to stimulate $CD4^+$ T helper cells, rather than by phagocytosis or receptor-mediated endocytosis in to the cytosol, the antigen or protein is taken up to endosomes where proteolysis occurs. The peptides enter a vesicle containing MHC class II where they bind and are transported to the cell surface [64].

DCs are derived from hematopoietic stem cells; however, they can originate from both lymphoid and myeloid lineages. In humans, myeloid lineage DCs ($HLA.DR^+/lin^-/CD11c^+$ - mDCs) are considered the "classical" DC. Myeloid DCs originate from myeloid committed

$CD34^+$ progenitor cells and monocytes. These cells can be stimulated to convert to mDCs in the presence of cytokines; GM-CSF, TNF, and IL-4 [63, 65-70]. These mDCs are capable of activating naïve CD4 and CD8 T cells, and inducing differentiation of naïve B cells to antibody secreting plasma cells [71]. By contrast, plasmacytoid DCs ($HLA.DR^+/lin^-/CD123^+$ - pDCs) originate from $CD34^+$ cells committed to the lymphoid lineage [65, 66]. These pDCs are $CD11c^-$ and are stimulated to become pDCs by IL-3 [72]. These DCs have the capacity to produce IFN-α, are closely involved in regulatory T cell and cytotoxic T cell activation, and reside in the T cell compartment of lymphoid tissues [73]. Finally, $CD16^+$ DCs ($HLA.DR^+/lin^-/CD11c^+/CD16^+$) are derived from monocytes, and activate T helper (Th) cells in an allogeneic fashion [65, 74]. Less is known about this miniscule population of mDC-related dendritic cells [71, 75]. Together, these cells play a crucial role in initiating and regulating both cellular and humoral immune responses.

1.4.9 Lymphocytes

T-cells and B-cells are lymphocytes involved in adaptive immunity, derived from the bone marrow lymphoid progenitor which bear variable cell-surface receptors used to identify and bind onto antigens (described below) [47]. T and B lymphocytes constitute approximately 20% of all circulating leucocytes and serve a wide variety of immune functions, including initiation of immune responses, production of cytokines and antibodies, cytotoxicity and memory of previous antigens [76].

Thymus-derived T cells account for approximately 70% of all lymphocytes (including NK cells) in the circulation. The major class of T cells is defined by its surface expression of the αβ TCR [47]. This receptor has evolved primarily to recognize peptide antigens presented in a complex with class I or class II MHC proteins [46]. αβ-T cells differentiate into several different subsets, $CD4^+$ T cells (predominantly T helper cells) or $CD8^+$ T cells (cytotoxic T cells) [47]. Cytotoxic T cells act primarily to kill cells infected with intracellular microbes, whereas Th cells act primarily to regulate the cellular and humoral immune responses. With only a few exceptions, the acquired immune response is initiated by APCs presenting an antigen to the Th cell within the peptide binding groove of MHC

class II molecules [45]. Primary functions of Th cells include forming a key part of the cell-mediated immune response, which involves cytokine secretions which activate further immune responses, and termination of infected cells by cytotoxic T-cells [49]. In addition to CD4 and CD8, T cells can be further defined according to the presence of CD45RA, CCR7, CD27, and CD28. As these cells mature, naïve T cells can differentiate into effector memory and central memory subsets. These subset changes reflect functional differences found under varying conditions. During active antigenic stimulation (e.g., during primary viral infection), effector memory phenotypes are able to eliminate viruses or tumours by different effector functions. In contrast, central memory T cells remain present in the absence of antigenic stimulation and have the capacity to expand rapidly upon secondary challenge [77].

Approximately 5–10% of T cells in the peripheral blood, lymph nodes, and spleen are $CD4^{-}CD8^{-}$. Some of these cells use αβ TCR and others use γδ TCR. γδ T cells have been labelled 'the bridge between innate and adaptive immunity', playing an important role in early immune responses to a variety of pathogens [78]. Displaying a wide array of functions including MHC-independent activation, T cell receptor-dependent recognition of antigens, and professional antigen presentation, while possessing protective memory and reactive memory capabilities [78, 79]. γδ T cells constitute only 1-5% of T lymphocytes in the blood stream in humans and mice [80]. These cells are, however, found in abundance in the epithelia of skin, genital, intestinal and respiratory tracts, highlighting their importance as an early responder to invasion [81-83]. Two major subsets (δ1 and δ2)—can be divided into four memory phenotypes (according to the presence of CD45RA and CD27)— known as naïve ($CD45RA^{+}/CD27^{+}$), central memory ($CD45RA^{-}/CD27^{+}$), effector memory ($CD45RA^{-}/CD27^{-}$) and terminally differentiated effector memory ($CD45RA^{+}/CD27^{-}$) phenotypes [84-87]. These memory phenotypes reflect functionally distinct populations based on cell maturity. Naïve cells have never encountered their cognate antigen and lack cytotoxic effector functions. Whereas, central memory, effector memory, and terminally differentiated effector memory cells are all antigen-experienced [85-88]. As these cells mature, characteristics present include greater cytotoxic capabilities and ability to migrate to sites of infection with a reduced lymph node migratory potential [85-91]. A number of cell surface receptors will be analysed on γδ T cells. These include CD11a, CD62L, and

CD94. The surface receptor CD11a is involved in cellular adhesion, and is typically up-regulated on endothelial cells in the proximity of inflamed tissues [92]. CD62L (also known as L-selectin) is also a cell adhesion molecule found on lymphocytes. Specifically, it functions as a "homing receptor" for lymphocytes to enter secondary lymphoid tissues via HEVs [93]. Essentially, CD62L expression reflects lymph node-migratory potential [86]. Finally, the CD94 receptor which is expressed primarily on NK cells and $CD8^+$ T cells (in addition to γδ T cells) is involved in innate recognition of aberrant MHC expression on infected and cancerous cells, used to stimulate or inhibit cytotoxic activity of these cell types[94, 95].

Finally, B-cells identified by expression of CD19, are involved in the secretion of vast amounts of immunoglobulins specific to the antigen that has initiated an immune response. This is part of the memory component of adaptive immunity [47].

1.4.10 Cytokines

Cytokines are a category of low molecular weight proteins (~5-20 kDa) that are important in cell signalling [49]. Cytokines act on cells via specific transmembrane cell surface receptors, typically by autocrine or paracrine signalling. Binding of the cytokine to the receptor elicits its cellular response by activating an intracellular signal transduction pathway that ultimately leads to induction of new gene transcription and synthesis of new cellular proteins [46]. Cytokines are involved in an array of processes in the body. They allow communication between various cell types and tissues, and are involved in the inflammatory response during exercise, trauma and infection. This involves the regulation by both pro- and anti-inflammatory cytokines. These cytokines can change in response to various stimuli (including exercise, trauma, infection, stress) and can influence the activity and function of other immune cells and tissues [96-98].

The immune system is regulated in part by circulating cytokines that change in concentration following various stimuli by paracrine cell-to-cell communication. Pro-inflammatory cytokines are up-regulated when stress occurs in the body, either during exercise, trauma or infection [96]. Major pro-inflammatory cytokines include interleukins (IL)-1, IL-6, tumour necrosis factor (TNF), and interferon-gamma (IFN-γ). The effect of

these pro-inflammatory cytokines on inflammation can be affected by anti-inflammatory cytokines including IL-1ra, IL-4, IL-6, IL-10, IL-11, and IL-13. They can attenuate inflammation by restricting pro-inflammatory cytokine production, up-regulating their soluble antagonist binding proteins, and suppressing inflammatory cell activity [99].

Activation of different immune response pathways during infection can be explained by the Th1 (T-helper cell ($CD4^+$) Type 1)/Th2 (T-helper cell ($CD4^+$) Type 2) cytokine balance (Figure 1.3). These T-helper cells regulate different immunological pathways. Resting naïve Th cells release very low levels of cytokines. Soon after stimulation by an antigen and APC, the Th cells begin to produce IL-2 and are designated Th0. As the Th cells continue to respond to the activating signal, they progress towards polar extremes of differentiation designated Th1, Th2, and Th17 depending on the nature of the cytokines present at the site of activation. The Type 1 pathway of cellular immunity is recruited to fight viruses and other intracellular pathogens, whereas the Type 2 pathway up-regulates antibody production to fight extracellular organisms as part of a humoral immunity response [49]. The Type 1 response involves pro-inflammatory cytokines where an APC presenting an intracellular pathogen releases IL-12 which act on NK cells, causing a release of IFN-γ. Together, IL-12 and IFN-γ act on naïve T-cells (Th0) to become Th1 cells. IFN-γ, IL-2, and lymphotoxin (previously known as TNF-β) are released from Th1 cells to activate the cellular immune response. These cytokines also act as a negative feedback mechanism to down-regulate the Th2 response. Conversely, the Type 2 response involves anti-inflammatory cytokines, where an APC presenting an extracellular pathogen releases IL-6 and IL-10, along with IL-4. This cytokine release drives naïve T-cells to become Th2 cells. IL-4, IL-10, and IL-13 are released from Th2 cells to activate the humoral immune response. These cytokines also act as a negative feedback mechanism to down-regulate the Th1 response [100]. This “balance” between these divergent pathways is important for maintaining immune health. Th1 cells drive the type-1 pathway to fight viruses and other intracellular pathogens, eliminate cancerous cells, and stimulate delayed-type hypersensitivity (DTH) skin reactions. Th2 cells drive the type-2 pathway and up-regulate antibody production to fight extracellular organisms. Over-activation of either pattern can cause disease, and either pathway can down-regulate the other [101].

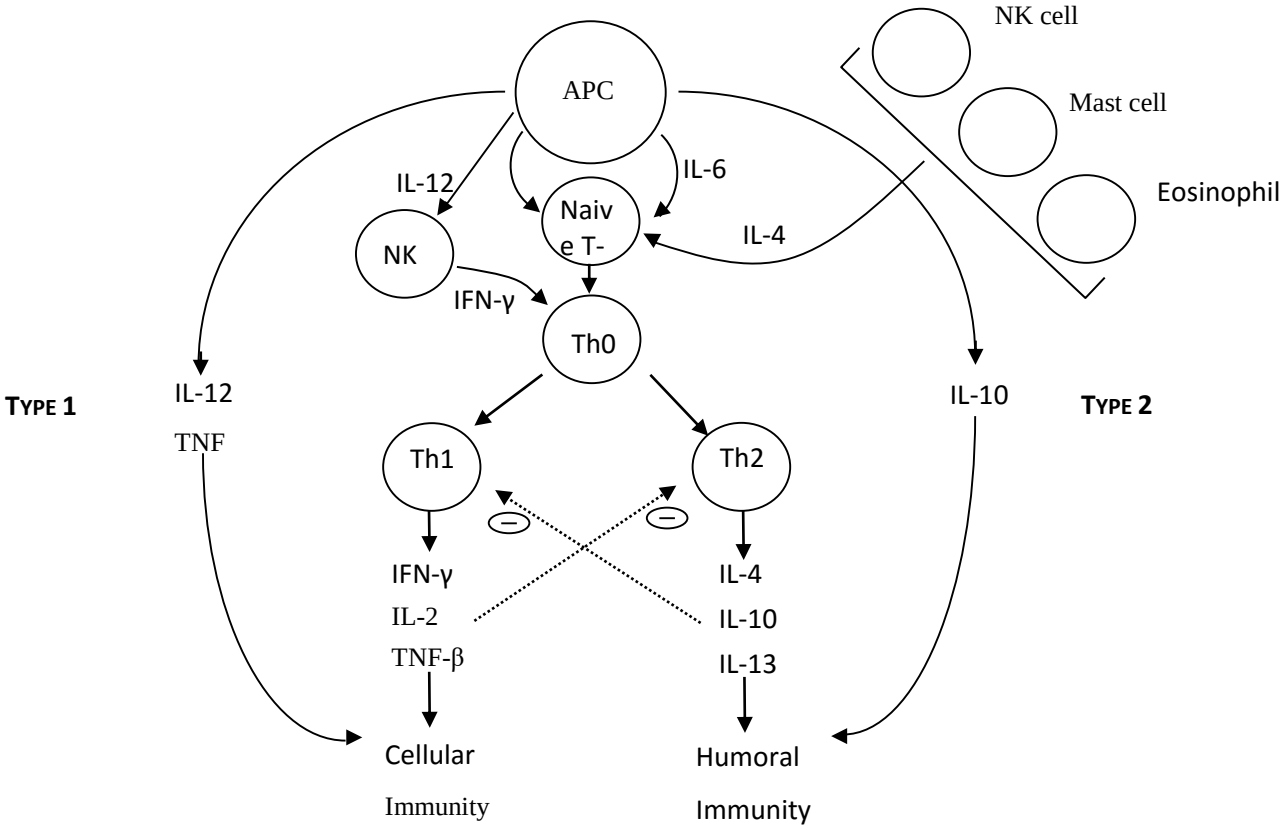

Figure 1.3. Th1/Th2 response (adapted from [100])

Apart from the Th1/Th2 response, T-helper cells can be differentiated into other cell pathways including the Th17 response. The Th17 subset is the first $CD4^+$ subset to be generated when mounting a response against infection. When APCs such as dendritic cells encounter a pathogen, a number of cytokines including IL-6, transforming growth factor (TGF)-β, and IL-23 are released, and act on naïve T-cells. The naïve T-cells then differentiate into Th17 cells, releasing IL-17A and IL-17E. These cytokines drive the release of chemokines; CXCL8, and CXCL2. This allows release of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor from epithelial cells. These chemokines recruit neutrophils directly from the lumen, whereas the colony-stimulating factors drive proliferation and differentiation of cells in the bone marrow, to produce a release of neutrophils and monocytes [49]. Together, these actions allow rapid response to early infection.

$CD8^+$ T cells also can manifest type 1 and type 2 cytokine responses, in which case the cells are designated T cytotoxic cell type 1 (Tc1) and T cytotoxic cell type 2 (Tc2).

1.5 Stress, Exercise and Immune Function

The stress-induced redistribution of leucocytes is a well-documented phenomenon [102]. A large portion of exercise immunology literature involves the characterization of changing cellular concentrations in the blood in response to various forms of exercise. For the purpose of consistency throughout this thesis, an increase in cell concentration of any leucocyte population in the blood following a stress response will be defined as cellular ingress into peripheral circulation. Furthermore, a decrease in cell concentration of any leucocyte population in the blood following a stress response will be defined as cellular egress out of peripheral circulation. These two phenomenon will collectively be described as cellular redistribution. An integrated definition of stress proposes that "*stress is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight or flight systems in the body (stress response)"* (reproduced with permission) [102, 103]. Previous research has highlighted a rapid ingress into peripheral circulation of neutrophils and lymphocytes in response to stressors such as psychological stress [104], exercise [1], and surgery [105]. This is regularly followed by a swift cellular egress [1, 106]. Specifically, ingress occurs by transportation of cells from areas of storage (i.e. spleen, lungs, marginated pool of respiratory tract, bone marrow, lymph nodes), into the circulation (i.e. blood vessels and lymphatics) and out to the tissues (i.e. skin, lung, gastro-intestinal and urinary-genital tracts, mucosal surfaces, and lymph nodes) [106, 107]. This stress response may (incidently) prepare immune cells to respond to infectious pathogens which may be imposed by the actions of the stressor [106, 108]. It has been suggested that the effect of stress on the immune system depends on the duration of the exposure to stress. Acute and intense stress may enhance immune function, and mild stress of moderate duration may promote immunosurveillance, while chronic stress may cause immune dysregulation [102, 109].

1.5.1 Leucocyte Demargination

There are a number of mechanism driving leucocyte redistribution during stress and exercise. While much of this thesis will discuss the role of neuroendocrine regulation of the immune system, it is important to mention the role of shear stress on changes in peripheral

blood leucocyte concentration. Leucocyte demargination is the process of cellular ingress into circulation as a result of increased blood flow (shear stress) [110]. In the early stages of acute exercise, leucocytes that were adhered to blood vessel walls, particularly those blood vessels in the lungs, spleen and the liver (marginated pool of leucocytes) become detached and enter the circulating pool. This is driven by both the shear stress of increased blood flow during exercise, and the actions of catecholamines (adrenaline and noradrenaline) [111]. The combined effects of shear stress and stress hormones drives a reduction in adhesion molecule expression on the cell surface of leucocytes and endothelial cells, making them 'less sticky', and increasing the concentration of leucocytes in circulation [112].

1.5.2 Neuroendocrine Regulation of Immune Function

Stressors such as exercise are characteristically met by a series of coordinated hormonal responses controlled by the central nervous system [113]. As mentioned above, the "flight or fight" stress response causes a cellular redistribution. Critical for mediating the effects of stress, the physiological stress response results in the release of neurotransmitters, hormones, peptides and other factors into the circulation or locally within tissues. The major mediators of stress effects are norepinephrine and epinephrine that are released by the sympathetic nervous system, and corticotrophin-releasing hormone, adrenocorticotropin, and cortisol, that make up the hypothalamic–pituitary–adrenal (HPA) axis [114]. A number of exercise and hormone infusion studies have elucidated several mechanisms driving cellular redistribution. As such, it has been hypothesized that just as the stress response prepares the cardiovascular, musculoskeletal and neuroendocrine systems for "fight or flight", under certain conditions, stress may also prepare the immune system for challenges (e.g. wounding or infection) that may be imposed by a stressor (e.g. predator or surgical procedure) [108]. This cellular redistribution is largely dependent on the concentration and duration of stress hormone release [115, 116]. These factors are dictated by the duration and intensity of the stress, the perception of the stimuli resulting in a stress response. Stress hormones release into the circulation is largely intensity-dependent. An exponential rise in plasma epinephrine, norepinephrine and growth hormone concentrations occurs with increasing exercise intensity [117-119]. Plasma cortisol

concentration, however has been reported to only increase above exercise intensities of 60% $\dot{V}O_2$max [120-122].

Catecholamines

The adrenal medulla is responsible for release of catecholamines; epinephrine and norepinephrine. Norepinephrine is also released by adrenergic neurons of the sympathetic nervous system. These two hormones are capable of affecting receptors in the cardiovascular, respiratory, gastrointestinal, and immune systems, in addition to endocrine glands, adipose tissue, and muscle. These catecholamines are strong responders to emotion stimuli and form the basis for the "fight or flight" hypothesis [123]. By binding to adrenergic receptors on target tissues, a stress-induced release of epinephrine and norepinephrine are hypothesized to prepare the body for danger. Adrenergic receptors can be further defined as alpha or beta (α_1 and α_2; β_1, β_2, and β_3). Through the secondary messenger mechanism binding of these hormones to different adrenergic receptors can illicit inhibitory or excitatory effects [124]. Changes in circulating concentrations of epinephrine and norepinephrine appear to be largely intensity-dependent. An exponential rise in plasma epinephrine and norepinephrine concentrations occurs with increasing exercise intensity [117, 119].

β-adrenoceptors are located on T-cells, B-cells, NK cells, neutrophils and macrophages with the exception of Type 2 T-helper cells [125]. During exercise epinephrine and norepinephrine plasma concentration's increase linearly with duration and exponentially with exercise intensity [126, 127]. Consequently, this hormone increase greatly affects the subsequent immune response. It has been suggested by Pedersen et al. (1997) that the original increase in circulating blood cell concentration of neutrophils and lymphocytes is due to epinephrine and norepinephrine. Evidence of this was observed following brief, intense or endurance exercise in which the concentration of circulating lymphocytes and neutrophils each correlated positively with plasma concentrations of epinephrine and norepinephrine [122, 128, 129]. Further evidence that catecholamines influence leucocyte ingress into circulation was observed by analysing the effects of increasing exercise intensity on catecholamine, lymphocyte, and granulocyte concentrations in the blood.

Furthermore, β-antagonist propranolol use during exercise has demonstrated that catecholamines regulate changes in circulating lymphocyte concentrations. Finally, activation of the stress response by exercise or β-agonist infusion have demonstrated a dose-dependent increase in lymphocyte concentration in peripheral circulation [104]. However, the increase in neutrophil concentration following infusion of catecholamines is significantly less compared to exercise−induced neutrophilia (increase in blood neutrophil concentration), suggesting further stimuli are inducing this increase in neutrophils during exercise [115, 130].

Cortisol

The primary glucocorticoid released from the adrenal cortex is cortisol. Cortisol contributes to the maintenance of plasma glucose during fasting and prolonged exercise [131]. The release of cortisol is driven by the following; corticotrophic-releasing hormone is secreted from the hypothalamus, causing the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into circulation. Binding to receptors on the adrenal cortex, ACTH increases cortisol secretion into the blood [132]. Cortisol is released during exercise to stimulate free fatty acid mobilisation from adipose tissue, mobilisation of tissue protein to yield amino acids for gluconeogenesis, and decrease the rate of glucose utilisation in cells [133]. The cortisol response to exercise appears largely due to exercise duration, where exercise under an hour rarely evokes a cortisol response [126]. There is a time lag response of cortisol, which generally peaks a number of hours after the peak in exercise-induced catecholamine release.

Compared to catecholamines, there appears to be a less consistent effect of cortisol on circulating leucocyte concentrations. Some studies report no relationship [128] or an inverse relationship [134] between plasma cortisol concentration and the number of circulating neutrophils after exercise. However, a number of studies have suggested cortisol concentration does alter neutrophil ingress into peripheral circulation following exercise [122, 135-137]. Additionally, infusion of cortisol causes lymphocytopenia, monocytopenia, eosinocytopenia and neutrophilia, peaking 4 h after administration [138, 139]. This assists in explaining both the incomplete neutrophilia in response to

catecholamine injection and the lymphocytopenia that occurs a number of hours after exercise.

Growth Hormone

Growth hormone (GH) is secreted from the anterior pituitary gland and exerts effects on growth of all tissues. GH secretion is controlled by growth hormone-releasing hormone and hypothalamic somatostatin, both released from the hypothalamus. Release of GH can by driven by exercise, stress, low plasma glucose concentration, and sleep [140]. Functionally, during exercise, GH assists in maintaining plasma glucose concentration, and is predominantly related to peak exercise intensity [141]. In response to an injection of GH at a dose corresponding to levels during exercise, a large neutrophilia occurred, although there were no effects on other leucocytes and cytokines [116]. However, there is no clear evidence to indicate that growth hormone regulates the number of circulating neutrophils following exercise [134].

1.5.3 Neutrophils

Exercise and other forms of stress are able to alter the concentration of neutrophils in peripheral circulation. A rapid, and profound neutrophilia during exercise is followed by a second, delayed increase in circulating neutrophil concentration a few hours later [142, 143]. This is variable between individuals depending on the exercise intensity, duration, and the training status of the individual undertaking the exercise. It is thought the initial neutrophilia is due to demargination caused by shear stress and catecholamines, whereas the later increase in neutrophil concentration several hours later may be due to release from the bone marrow due to an rise in cortisol concentration post-exercise [128].

During exercise, the concentration of circulating leucocytes increases substantially, primarily due to an increase in neutrophil concentration. This is known as neutrophilia, and is common among all forms of exercise, but the degree of change in neutrophil concentration is dependent on exercise and subject characteristics such as training status [76]. Changes in circulating neutrophil concentration may significantly alter both the

immune and inflammatory responses. Neutrophil concentration increases following bouts of strenuous exercise such as endurance competition [144-147], during training sessions [148-152], and in predefined training conditions at a set threshold under laboratory conditions [1].

In a Japanese study, endurance-trained runners were monitored following a competitive marathon. All subjects (age 36.6 ± 5.1 years; body mass 60.4 ± 4.2 kg) had completed a previous marathon under 2 h 40 minutes. Neutrophil concentration increased significantly immediately following exercise [153]. Sampling points were only conducted before and immediately after exercise, limiting the data available to monitor the post-exercise immune recovery. In another study involving 14 male competitive cyclists and triathletes (age 25 ± 5 years; body mass 72 ± 9 kg; $\dot{V}O_2$peak 67 ± 6 $mL.kg^{-1}.min^{-1}$) who cycled for 4 h on a 400 m track at a given workload of 70% of the individual anaerobic threshold, neutrophil concentration increased significantly immediately after exercise, remained elevated for 1 h, and returned to resting levels by 19 h after exercise [150]. Similarly, in eight highly trained cyclists (age 38 ± 4 years; body mass 72.5 ± 7.0 kg; $\dot{V}O_2$peak 61.0 ± 8.8 $mL.kg^{-1}.min^{-1}$) who exercised on a cycle ergometer at an average of 100 W for 45 minutes, a significant increase was observed in neutrophil concentration from before to immediately after exercise. It is unknown when neutrophil concentration returned to the resting level due to lack of post-exercise sampling points [154]. In a study of 17 professional cyclists (age 22.3 ± 0.9 years; body mass 70.6 ± 1.3 kg; $\dot{V}O_2$peak 81.9 ± 2.6 $mL.kg^{-1}.min^{-1}$), circulating neutrophil concentration increased significantly from before to immediately after a 137.1 km cycling race. At 3 h after exercise, neutrophil concentrations significantly decreased from immediately after exercise but were still significantly elevated above pre-exercise levels [145]. Finally, in ten highly trained male cyclists (age 24.2 ± 5.3 years; body mass 73.8 ± 6.5 kg; $\dot{V}O_2$peak 65.9 ± 7.1 $mL.kg^{-1}.min^{-1}$) who exercised for 2 h at approximately 75% $\dot{V}O_2$peak, neutrophil concentration increased significantly from before to immediately after exercise, and increased further at 2 h after exercise. Samples were taken until 8 h after exercise (at 2 h intervals), at which point they were still significantly elevated above pre-exercise (resting) levels [1]. This showed that neutrophils have not returned to pre-exercise resting levels at 8 h after exercise, which potentially may have implications for immune

cell redistribution if a second bout of exercise were to be undertaken within this 'recovery' period.

1.5.4 Lymphocytes

Circulating lymphocyte concentration has a biphasic response to prolonged exercise [61]. During and immediately after an acute bout of exercise, a lymphocytosis (increase in circulating lymphocyte concentration) is observed. However, after exercise, lymphocyte concentration decreases below pre-exercise levels for approximately 2–4 hours post-exercise [155], before steadily returning to resting values. This response varies depending on exercise intensity, duration and fitness of the subject [156, 157]. Many studies have found this biphasic effect during and after exercise (approximately from immediately after to 4 hours after exercise) for, T lymphocytes, B lymphocytes, and Natural Killer cells [1]. Ingress of lymphocytes into peripheral circulation is largely driven by the actions of epinephrine, both directly by increasing expression of cell adhesion molecules, and indirectly via sympathetically activation resulting in increases in cardiac output and shear stress associated with enhanced blood flow [158].

A recent study monitored immune response at pre-exercise, immediately post-, 2 h, 4 h, 6 h, 8 h, and 24 h post-exercise in highly trained cyclists (age 24.2 ± 5.3 years; body mass 73.8 ± 6.5 kg; $\dot{V}O_2$peak 65.9 ± 7.1 mL.kg^{-1}.min^{-1}). Following 2 h of exercise at approximately 75% $\dot{V}O_2$peak the biphasic response of circulating lymphocytes did occur. This featured a significant increase in lymphocyte concentrations from before to immediately after exercise, followed by a significant decrease from immediately after to 2 h after exercise. Lymphocyte concentration then significantly increased again to above pre-exercise levels by 6 h after exercise, and remained elevated to 8 h after exercise, only returning to resting levels at 24 h after exercise [1]. These results indicate that lymphocyte concentration had not returned to resting levels 8 h after exercise, potentially having implications for immune function if a second bout of exercise were to be taken within this 'recovery' period.

Separating lymphocytes into their respective subsets reveals an interesting relationship with the stress response. The greatest expression of β-adrenergic receptors is found on the

surface of NK cells, followed by γδ T cells, then $CD8^+$, and least of all on $CD4^+$ cells [104, 158]. The difference in effects of exercise on the relative magnitude of ingress of the lymphocyte subsets into circulation will be discussed below, and reflects this differential density of adrenergic receptor expression on the surface of these cell types.

$CD4^+$ and $CD8^+$ T cells

While $CD4^+$ and $CD8^+$ T cell redistribution won't be the focus of this study, it is important to consider their functional characteristics and understand their role in the larger scheme of the stress response. It is well established that, $CD4^+$ T helper cells, along with a number of other cells, are responsible for regulating the T helper cytokine responses, whereas, $CD8^+$ cytotoxic T cells are predominately involved in termination of virally infected cells. As such, it is important to consider their role in and response to stress and exercise. Previous research has demonstrated a repeatable ingress of both $CD4^+$ and $CD8^+$ T cells into peripheral circulation, similar to that of total lymphocyte concentration. A slightly more aggressive ingress into circulation has been observed in $CD8^+$ compared to $CD4^+$ T cells [104] following a stressor, reflecting the β-adrenergic receptor density on the surface of these cell types [158]. This ingress was observed following psychological stress, low- and high-intensity, and prolonged exercise [104, 155]. Furthermore, β-agonist infusion also drives ingress of $CD8^+$ but not $CD4^+$ T cells [104]. As with total lymphocyte concentration, a biphasic change in the circulating concentration of $CD4^+$ and $CD8^+$ T cells is present. Interestingly, the decrease in T cell concentration after exercise is largely due to a decrease in the concentration of Type 1 T cells, while little effect is observed on changes in the percentage of circulating Type 2 T cells [155, 159].

γδ T cells

Previous research has demonstrated that acute exercise and psychological stress drive ingress of γδ T cells with high cytotoxic capabilities, tissue homing potential, and the capacity for rapid, innate-like target recognition [104, 160]. A significant increase in total γδ T cell concentration in peripheral circulation [104, 161, 162], and both δ1 and δ2 subsets [163], following short duration exercise (30 min at 35%W_{max} and 85%W_{max}), and

in response to psychological stress and β-agonist infusion has been demonstrated. When examined for surface receptors; CD11a, CD62L, and CD94 on γδ T cells, those cells featuring high cytotoxic capabilities ($CD94^+$), tissue homing potential ($CD11a^+$) were mobilised to a greater extent following psychological stress [163]. No research has examined this population, highly sensitive to stress, followed prolonged, heavy-intensity exercise, or in response to repeated exercise bouts.

1.5.5 Dendritic Cells

Few studies have been published regarding cellular redistribution of dendritic cells. Following a marathon run, mDC circulating concentration increased significantly, whereas pDC concentration in the blood decreased significantly below pre-exercise values immediately post-race [164]. Furthermore, following a 1-hour ice-hockey training session, concentrations of mDCs and pDCs in the peripheral circulation both increased significantly compared with rest after the session was completed [165]. Finally, a significant increase in total DC concentration in the blood was observed following surgery or an exercise stress test to exhaustion [105]. No studies to our knowledge have examined changes in DCs under conditions where exercise intensity and duration are controlled, nor in response to repeated exercise bouts.

1.5.6 Th1/Th2 relationship

One study has examined changes in the Th1/Th2 relationship to an acute bout of endurance exercise. Nine male runners (age 30 years; body mass 74.5 kg; $\dot{V}O_2$peak 60.1 $mL.kg^{-1}.min^{-1}$) ran on a treadmill for 2.5 h at 75% $\dot{V}O_2$peak during which blood samples were taken before exercise, 30 min into exercise, immediately after, 2 h and 24 h after exercise. There was a significant decrease in the proportion of Th1 cells in the blood (percentage of IFN-γ and IL-2 producing $CD3^+$ cells) whereas no change was found in the proportion of Th2 cells (percentage of IL-4 producing $CD3^+$ cells) [155]. This change in circulating proportions of Th1 and Th2 cells may have implications for the function of different immune cells. It is unclear whether these changes are due to apoptosis or, as seems more likely, egress of cells out of peripheral circulation to other compartments. A decrease in the

percentage of Type 1 $CD4^+$ and $CD8^+$ T cells alone does not necessarily indicate that defence against intracellular pathogens such as viruses is suppressed [111]. Importantly, glucocorticoids and catecholamines appear to influence Th cytokine release, principally by stimulating glucocorticoid and β2 adrenergic receptors, respectively [166]. In turn, these stress hormones influence the activity of cellular (Th1), humoral (Th2), and mucosal (Th17) branches of the immune response [166, 167]. Specifically, cortisol, epinephrine and norepinephrine may suppress cytokine release from APCs and Th1 cells, while up-regulating cytokine production from Th2 cells, thereby causing a shift toward Th2 cytokine production [155, 168]. Further research is warranted to examine the effects of stress and exercise on the T helper cytokine response. Additionally, only one study has examined the Th17 cytokine response to exercise [169]. This study examined the effect of strenuous endurance exercise (marathon and half-ironman triathlon) on $CD4^+$ lymphocyte subpopulations including Th17 cells. Endurance exercise induced a significant increase in Th17 cell concentration immediately post-exercise. Conversely, no study has examined the effect of exercise on Th17 cytokine production. More research is warranted as Th17 cells are involved early on in regulation of immune function during infection, and may potentially be compromised by the exercise−induced stress response.

1.5.7 Stress Hormone/Cytokine Interaction

The Th2 shift that is reported to occur during exercise can be explained by the stress hormone–T-helper cell interaction (Figure 1.4). This process is driven by catecholamines and cortisol, and by stimulation and inhibition of APCs and T-helper cells [170].

Catecholamines act on the APCs and Th1 cells. APC release of IL-12 is suppressed by catecholamines, while release of IL-10 is increased [170]. Furthermore, Th1 cells are inhibited by catecholamines binding to β-adrenoceptors [171], while β-adrenoceptors are not expressed on Th2 cells [172]. These combined processes down-regulate differentiation of Th1 cells, as well as blocking inhibition of Th1 and APC-produced pro-inflammatory cytokines, which inhibit the Th2 pathway (IL-2, IL-12, IFN-γ and TNF).

Cortisol suppression of the Type 1 pathway occurs through a number of mechanisms. Previous studies have shown that cortisol decreases the production of TNF, IFN-γ, IL-2

and IL-12 from APCs and Th1 cells [173, 174]. This down-regulation blocks the inhibition of pro-inflammatory cytokines in the Th1 pathway, resulting in an increase in IL-4 production [175]. Additionally, cortisol causes a decrease IL-12 receptors on the cell surface of NK and T cells [176].

The combined effects of catecholamines and cortisol cause a shift of the Th1/Th2 balance towards Th2, causing a release in anti-inflammatory cytokines and further enhancing humoral immunity. This may have implications for suppression of the cellular immune system in the period following exercise. However, there is limited research examining the immune response to exercise and its influence on Th1/Th2/Th17 cytokine regulation.

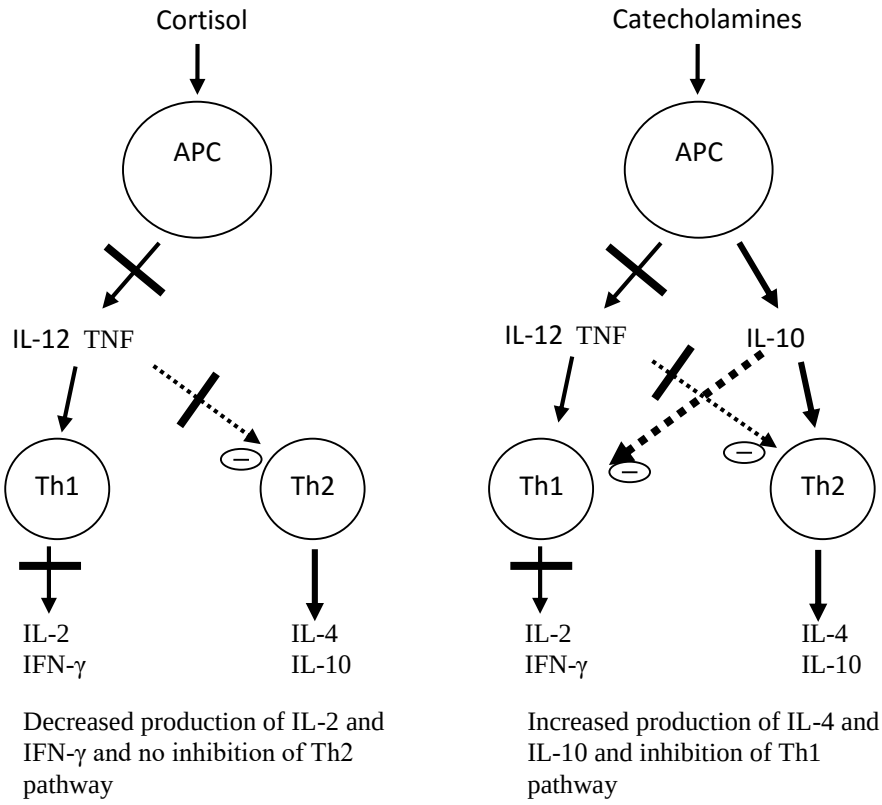

Figure 1.4. Stress hormone effects on Th1/Th2 cytokine release (adapted from [100])

1.5.8 Repeat Bouts of Exercise

Elite athletes undergo strenuous training programs sometimes involving multiple sessions of conditioning per day. Some previous research has examined immune system function

following successive exercise bouts [156, 177-188]. Of limited number of studies involving repeated exercise, a number examined exercise duration and intensity or recovery duration not comparable to the repeated prolonged heavy-intensity exercise focus of this thesis [180-185]. Below are those studies which fit within the context of this thesis.

The effect of repeated exercise was measured in 10 healthy males (age 21.6 ± 0.4 years; body mass 78.0 ± 4.6 kg; $\dot{V}O_2$peak 54.0 ± 2.7 mL.kg^{-1}.min^{-1}) who exercised for 1 h sub-divided into three 20 min segments (5 min at 50% $\dot{V}O_2$peak and 15 min at 70% $\dot{V}O_2$peak) on two occasions separated by 4 h. Both circulating neutrophil and lymphocyte concentrations were significantly higher after two bouts of exercise than after the single bout and NK cytotoxic activity was significantly higher following the second bout [187]. Two more studies monitored nine endurance trained athletes (age 21–27 years; body mass 74.7 ± 5.4 kg; $\dot{V}O_2$peak 69.1 ± 3.7 mL.kg^{-1}.min^{-1}) who completed one or two bouts of cycling for 75 min at ~75% $\dot{V}O_2$peak separated by a 3 h rest period. Following the second bout of endurance exercise, there was a significant increase in circulating concentrations of neutrophils, lymphocytes, $CD4^+$, $CD8^+$, and $CD56^+$ cells, epinephrine, norepinephrine, ACTH, cortisol, and growth hormone, and a significant decrease in testosterone compared to a single bout of endurance exercise [177, 179]. Furthermore, in the same subject group, two studies were undertaken involving the same exercise protocol, however one group was given a 6 h rest period rather than 3 h. A significantly larger neutrophilia and lymphocytopenia was seen in the group with the smaller rest period following the second bout of exercise. Additionally, interleukin-6 (IL-6) and interleukin-1 receptor antagonist (IL-1ra) circulating concentrations were found to be significantly higher following exercise with 3 h rest compared to 6 h [178, 189].

A number of these studies have highlighted a ‘carry-over’ or ‘additive’ effect of repeated exercise bouts on lymphocyte and neutrophil concentration changes in the peripheral circulation [177-179, 186-188]. These carry-over effects of repeated exercise have been hypothesised to lead to chronic suppression of immune function [76]. In concert with a pronounced peak in plasma catecholamine and cortisol concentrations during exercise, the resultant ingress of lymphocytes and neutrophils into circulation is more severe during and after repeated exercise [177, 179]. However, cellular ingress only describes half the story.

It is recognised that following stress-induced cellular ingress, egress of these cells out of peripheral circulation occurs [104]. This egress of cells involves movements to areas at greater risk of potential infection, such as the epithelial surfaces of skin, gastrointestinal, urinary, and respiratory tracts [106]. It is largely unknown how prior intense exercise alters the cellular immune system during and following a subsequent exercise bout. Presently, only neutrophils and lymphocytes (including $CD4^+$, $CD8^+$ and $CD56^+$ cells) have been examined within this context [177-179, 186-188]. No research has investigated the effects of repeated bouts of exercise on a number of lymphocyte subsets or dendritic cells in the circulation. This may cause a compounding effect on the immune system, potentially increasing the severity of the exercise-induced stress. This is important to consider in the context of an increased rate of symptoms associated with URIs during periods of competition and heavy training [8, 13, 14, 26-28].

1.5.9 Saw-Tooth Theory?

Expanding on the concept of a 'carry-over' or 'additive' effect, a 'saw-tooth' theory has been put forward, hypothesising that if a single bout of exercise might cause a suppression of the immune system, will repeated exercise without adequate recovery cause greater immune suppression? This theory dates back to when the 'open window' theory was at the forefront of hypotheses in exercise immunology [76]. It has been suggested by Mackinnon (1999) that the cumulative effects of repeated exercise without complete recovery of the immune system may eventually lead to a chronic suppression, possible increasing the risk of URI in such athletes (see Figure 1.5). We now know that the increased rate of URIs is more complicated than a single variable, however it is still worth considering the influence of repeated exercise-induced stress without sufficient recovery. This is because an increased rate of symptoms associated with URIs are found predominately during periods of competition or heavy training [8, 13, 14, 26-28].

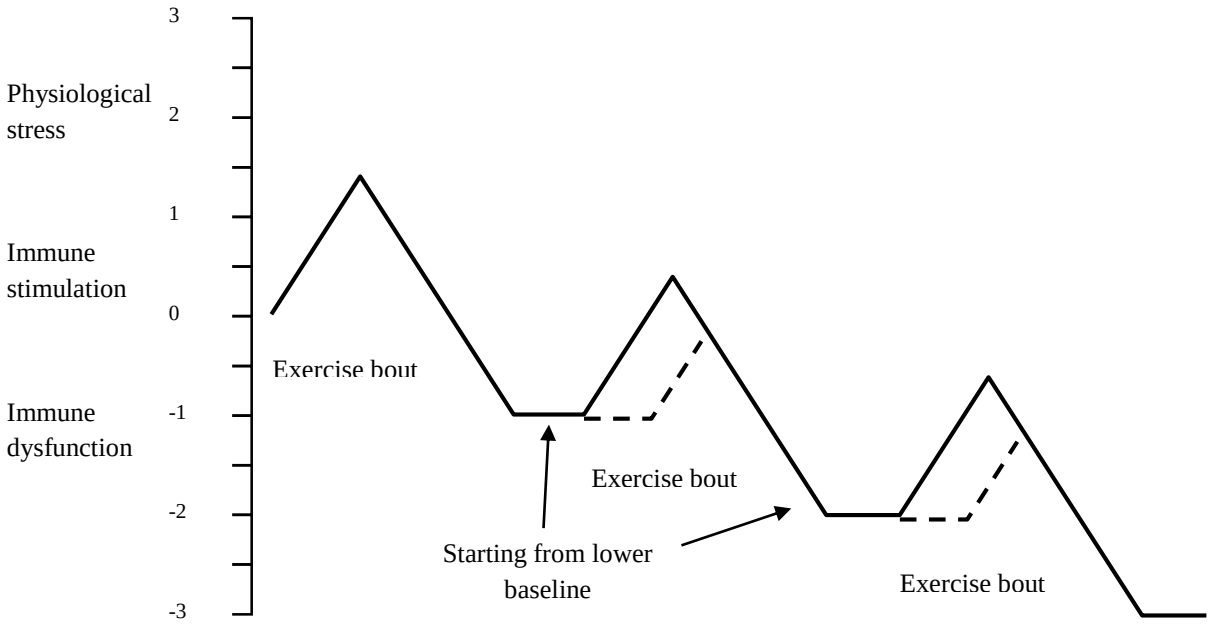

Figure 1.5. Model: Variation on 'open window' with repeated exercise (adapted from [76])

1.5.10 Influence of Feeding on the Immune System

One variable bearing large influence on plasma cortisol concentration during exercise, is the presence or absence of a feeding protocol during the sample collection period. As previously discussed, leucocyte redistribution is dictated largely by plasma catecholamine and cortisol concentrations [104, 122, 128, 135-139]. Complicating matters, these stress hormones are heavily involved in maintaining blood glucose concentration at a normal homeostatic level during exercise and fasting [133, 140, 190]. Previous research has also demonstrated that inducing a stress response on subjects' while in a fasted state results in no increase in salivary cortisol concentration, while a significant increase is observed in fed subjects [191, 192]. Salivary cortisol has a high correlation coefficient ($r = 0.893$) with plasma cortisol concentration [193]. Furthermore, while feeding appears to increase cortisol concentration [194], feeding during exercise appears to decrease it (compared to placebo) [195, 196]. Certain protocol designs may accentuate this effect, particularly in protocols including sampling for many hours after the end of exercise. While some studies did not feed their subjects for the duration of a sample collection protocol [186, 197], others have done the opposite [1, 177-179, 189, 198]. Importantly, in those studies with no

feeding permitted, subjects were without food for a maximum of twenty hours (including the overnight fast) and completed one or two prolonged heavy-intensity exercise bouts. Together, these two factors may have had a large influence on changes in leucocyte distribution during exercise and the post-exercise recovery period. Potentially, this effect may be further exacerbated in repeated exercise protocols. Previous research examining repeated, prolonged, heavy-intensity exercise effects on immune cell redistribution has highlighted that subjects were not adequately replenished to allow fulfilment of a repeated exercise protocol [186]. This was likely due to combined effects of the depletion of carbohydrate stores, and an exercise intensity that would require the consumption of such a substrate. Of course, control of feeding is employed to limit variables when designing a study. However, this protocol design does not reflect recommended post-exercise recovery protocols, and may result in changes which would not occur during optimal training.

1.5.11 Other Factors Affecting Immune Response to Acute Exercise

Although not the direct focus of this thesis, it is important to discuss a number of factors that can affect immune response to acute exercise. Specifically, infection history, age, gender, and certain environmental factors can affect how a person's immune system responds to a bout of exercise.

Infection history can affect the immune response to exercise. Specifically, viral reactivation can have an impact on the rate of presentation of URI-associated symptoms in athletes. Persistent reactivating herpesviruses that are prevalent among humans include herpes simplex virus (HSV-1, HSV-2), Epstein-Barr virus (EBV), varicella zoster virus (VZV), and cytomegalovirus (CMV) [199]. One study examining the profile of infections of elite athletes experiencing recurrent URS associated with long-term fatigue and poor performance identified a high percentage as having herpes group viruses (e.g. CMV) or evidence of EBV reactivation [17]. Epstein Barr viral reactivation has also been demonstrated in association with URS in some endurance sports [17, 24].

Immunosenescence is the overarching term that has been used to describe the biological ageing and progressive dysfunction of systemic immunity. This age-related diminution of the immune system is characterized by poor vaccine efficacy, lowered immune vigilance,

and greater morbidity and mortality as a result of infectious disease [200, 201]. This senescence is associated with reactivation of herpesviruses.

There remain a number of gender difference in the immune-endocrine response to exercise [202]. Although data are limited, some sex-based differences in immune responses to endurance exercise have been observed [203, 204]. Women have shown a greater lymphocyte response to prolonged cycling exercise than men [204], although this may be specific to woman who are physically trained since gender does not appear to influence lymphocyte apoptosis in response to maximal endurance exercise in untrained subjects [205]. Furthermore, women also demonstrate an attenuated inflammatory response to muscle damaging exercise than men, even when muscle damage is similar [206]. While limited studies exist, there appears to be some gender differences in the immune system response to exercise.

Environmental factors can influence the way the immune system responds to acute exercise. Potential adverse conditions include extremes of heat and humidity, cold, high altitude, and air pollutants, although it remains a controversial suggestion that physical activity performed in stressful environments poses a greater than normal threat to immune function [207]. Exercising in hot versus thermoneutral conditions (core temperature ≥1°C higher in hot conditions) augments circulating stress hormones, catecholamines, and cytokines with associated increases in circulating leukocytes [208, 209]. However, exercise in the heat appears to elicit limited effect on neutrophil function, monocyte function, natural killer cell activity, and mucosal immunity [207, 210-217]. Therefore, most of the available evidence does not support the argument that exercising in the heat poses a greater threat to immune function (vs thermoneutral conditions). However, due to ethical committee restrictions, most laboratory studies have evoked modest core temperature responses (<39°C), rather than what can be reached during exercise in the field (~39.5°C) [218], or during heat stroke (core temperature >40.6°C) [219]. Studies investigating the effects of cold and high altitude on immunity and infection incidence are often hindered by extraneous stressors (e.g. isolation) [207]. Of the available evidence, previous research does not support the popular belief that short- or long-term cold exposure, with or without exercise, suppresses immunity and increases infection incidence [134, 211, 212]. Some evidence shows that ascent to high altitude increases infection incidence [220-222]. Studies

have reported suppressed cell-mediated immunity in mountaineers at high altitude [221]; however, the impact of this finding on resistance to infection remains unclear. When designing studies in exercise immunology, it is necessary to consider the environment in which participants are undertaking exercise, as the immune response can be altered by altitude, temperature and humidity; to what degree remains a contentious issue.

1.6 Flow Cytometry

Flow Cytometry is a laser-based biotechnology that utilizes light scattering and fluorescence emission to quantitatively analyse the properties of individual particles, such as cells. The flow cytometer is capable of passing suspended cells in a stream of fluid through a laser [223], demonstrated in Figure 1.6. Quantitative measurement of parameters is obtained by several systems working in unison. A single stream of particles is transported and focused by a hydrodynamic fluidics system. This stream is guided through a laser beam for interrogation [223]. The biological sample undergoing analysis is injected into a stream of sheath fluid, located in the flow chamber. Differences in the density and velocity of the two fluids allow them to form a two-layer stable flow (laminar flow) that does not interact [223]. Difference between the velocities of flow of the two fluids allows the sample fluid to be restricted to the centre of the flow chamber. This control of the fluids allows suspended cells in the sample fluid to be directed through the laser beam. In addition to the laser, the optical system consists of a series of focusing lenses which direct light generated following interaction of the laser with particles in the flow stream, and a series of dichroic mirrors and band-pass filters which direct specific wavelengths of light to the appropriate photo-detector for signal processing [223, 224]. A photo-detector produces a small current when light is detected from sample particles interaction with the laser. The amplitude of the current is proportional to the total number of light photons received by the detector [223]. Via a series of linear and logarithmic amplifiers, the electrical current is amplified and then converted to a voltage pulse. This voltage pulse is assigned a digital value by the Analog-to-Digital Converter (ADC). Each voltage pulse generated represents data from a single particle. Within the range of 0-1000mV, the ADC converts the pulse to a digital number representing 0-1000mV channels [223]. Data is stored and displayed via computer software to allow for analysis and comparison of samples [223, 224].

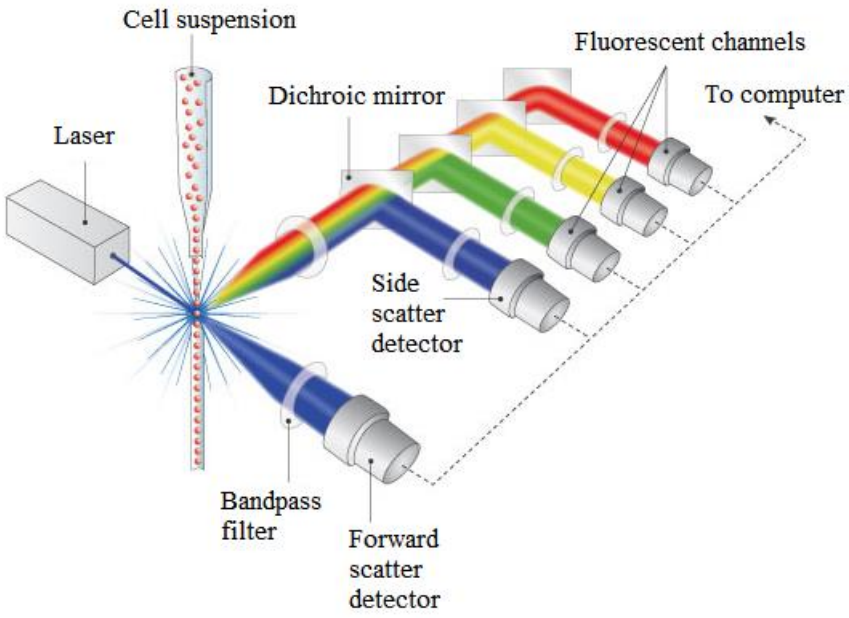

Figure 1.6. Model: A diagram illustrating the fluidics and optical systems of a flow cytometer

1.6.1 Light Scatter

In the context of cell counting and sorting, light scatter is paramount in identifying populations of cells. When sample particles are passed through the laser beam, deflected laser light scatters in all directions. The forward scatter channel (FSC) collects light scattered in a forward direction. It is measured at an approximate 20^{o} offset from the axis of the laser beam by a type of photo-detector called a photodiode. Light collected by the FSC is proportional to a particle's surface area and equates to a particle's size [223]. Conversely, side scatter light reflects cellular granularity or the internal complexity of sample particles. This side scatter light collected in the side scatter channel (SSC), is measured perpendicular to the axis of the laser beam, by a photomultiplier tube detector [223].

1.6.2 Fluorescence

Fluorescence is used in flow cytometry to identify specific proteins on the surface, or located inside of cells, or found in solution via the use of fluorochromes. Travelling in

waves, light is a form of electromagnetic energy [225]. The energy content of a light beam is what determines the colour of light. Additionally, this energy content is directly proportional to the light beam's wave frequency and inversely proportional to its wavelength [225]. A fluorescent compound is capable of absorbing light energy within a specific wavelength range. This absorption of light causes excitation of the electrons within that compound, raising the electrons to an unstable higher energy state [223]. Some energy is then released as heat by the fluorescent compound, undergoing a conformational change to return to a more stable state [223]. The subsequent release of the remaining energy as light, emitted at a longer wavelength to the light source which originally excited the compound, is referred to as fluorescence [225].

In addition to identifying forward and side scatter characteristics of cells, lasers are used to focus light consisting of a single wavelength on the individual particles of a sample [223-225]. As mentioned above, fluorochromes are used to analyse specific cellular parameters. These fluorochromes are maximally excited by the flow cytometer within a specific wavelength to target specific cellular antigens or intracellular molecules. Light emitted by these fluorochromes allows for the quantification of the biological and biochemical properties of the target molecule. This is because the amount of fluorescent signal detected by the flow cytometer is proportional to the number of fluorochrome molecules on the particle [223, 225]. Flow cytometers use separate fluorescence channels to detect the specific wavelength of light emitted from fluorochromes allowing for multiple identifying markers to be analysed in a single sample.

1.6.3 Data Analysis

Computer software allows the data collected above to be graphed and analysed statistically [223, 224]. Histograms, dot plots, contour plots or density plots can be used to visualise one or two parameters at once. This visualisation of data can facilitate the identification of various cell populations which are associated with specific antibodies, or forward or side scatter characteristics [223].

During the data collection process, digital conversion of a light scatter or fluorescent signal can be processed by either linear or logarithmic amplification of the electrical current

produced by a photo-detector [223]. Linear data is reported on a 1024 channel scale in proportion to the intensity of collected light and displayed graphically on axes with linear values. This linear scale is used for analysis of parameters in which a narrow range of signal intensity is expected, such as forward scatter and side scatter light [226]. Conversely, the logarithmic amplified data is reported on a 1024 channel scale with the digital channel number proportional to the log of the original light intensity. This logarithmic amplification is normally used to display data of fluorescent parameters as it compresses strong signals which range up to hundreds or thousands of times the intensity the intensity of weak signals, allowing such data to be displayed graphically [223, 226]. Analysis of the relative intensities of logarithmically amplified signals is achieved by converting the channel values to a number that corresponds to the fluorescent intensity of the original light signal. All values are displayed graphically on a four log decade scale ($10^0 – 10^4$) corresponding to linear values in the range of 1 – 10,000 [227].

Cells are often analysed for the presence or absence of a given marker to better define and categorise the cell type [228]. A negative control sample is required to accurately distinguish between positive and negative data sets [228]. Negative isotype controls are commonly used as control samples, providing non-specific staining for an antibody of a particular isotype conjugated to a particular fluorochrome and biological comparison controls. This provides relevant comparison conditions to determine the presence or absence of a given marker [228].

Analysis of different cell populations can be achieved by analysing both forward and side scatter, and the presence or absence of conjugated fluorochromes. This can be assisted by the use of ‘gating’. Computer software allows for subsets of collected data to be analysed in isolation. Numerical or graphical boundaries are created by “gates”. “Gates” are utilised to identify cells of interest while excluding data from unwanted cells [223]. These boundaries define the characteristics of particles to be included in further analysis [223]. This is used to target specific cell populations [223]. Granulocytes, monocytes and lymphocytes can be visualized by graphing forward scatter and side scatter parameters on a dot plot. Once gated according to size and granularity, identification of specific CD molecules can be used to further define different cell types.

In addition to cellular identification, flow cytometry can be used in protein quantification. A cytometric bead array (CBA) allows for the quantification of multiple proteins simultaneously [229]. In general, a CBA assay consists of a mixture of beads uniform in size (7.5 μm) but containing different fluorescence intensities of a red-emitting dye. A different capture antibody against each specific protein (such as a cytokine) is covalently coupled to each type of bead. Cytokines bound to these antibodies are detected by use of antibodies labelled with phycoerythrin. The fluorescence intensity measured with phycoerythrin is proportional to the cytokine concentration in the sample and is quantified from a calibration curve [230]. CBA provides an adequate supplement to an enzyme-linked immunosorbent assay (ELISA), allowing for multiple analytes to be quantified in a single sample.

1.7 Mitogen Stimulation

Mitogen stimulation is used for *in vitro* assessment of cellular immunity for research purposes, in addition to examining patients with immunodeficiency [231], autoimmunity [232], infectious diseases [233], and cancer [234], reflecting immune function *in vivo*. Stimulating isolated cells allows a functional representation of a cellular population at rest, or while under pre-determined controlled conditions. Common mitogens used for stimulation include phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA) and ionomycin, and anti-CD3/anti-CD28 coated beads [235, 236].

1.7.1 Phytohaemagglutinin

Phytohaemagglutinin is a lectin extracted from red kidney beans (*Phaseolus vulgaris*) [237, 238]. The protein consists of two molecular species, a leucoagglutinin (PHA-L) and an erythroagglutinin (PHA-E) [239, 240]. PHA-M is the mucoprotein form and is a crude extract used for the stimulation of cell proliferation and cytokine production in *in vitro* lymphocyte culture for subsequent detection in immunoassays, either by ELISA [241], cytometric bead array [242, 243], intracellular cytokine identification [241]. PHA can also be used as a stimulant in leucocyte proliferation assays [244].

Although, a large amount of literature exists on PHA-stimulation of immune cells to induce proliferation following exercise [244-253], few studies have examined PHA-stimulated cytokine production following prolonged exercise. Of those exercise-related studies, a PHA stimulation assay was used to analyse T helper cytokines (TNF, IFN-γ, IL-10, IL-6, IL-4, and IL-2) released from isolated PBMCs of endurance athletes [242]. Blood samples were collected at rest and incubated with or without the addition of bovine colostrum. Cytokines were quantified by cytometric bead array. The results demonstrated bovine colostrum is able to modulate *in vitro* cytokine production of human PBMCs [242]. Of those studies examining PHA-stimulation of PBMCs following exercise, conflicting results are apparent. In one study, IFN-γ concentration significantly increased from pre-exercise to 24 h following moderate, but not exhaustive exercise, whereas no significant change was reported in IL-2 concentration [254]. Furthermore, no significant change was apparent in IFN-γ or IL-2 production following PHA-stimulation of PBMCs after cycling for 60 min at

75 % of $\dot{V}O_2$peak [255]. Conversely, following cycling for both, 60 min at 60 % of $\dot{V}O_2$peak, and a 19 min exhaustive effort, PHA-stimulation of PBMCs resulted in a significant decrease in IL-2 production [250, 256]. To our knowledge, no other cytokines have been analysed following PHA-stimulation of PBMCs following prolonged, heavy-intensity exercise.

1.7.2 Phorbol 12-myristate 13-acetate & ionomycin

Another common cell stimulation cocktail used in immunology research combines phorbol 12-myristate 13-acetate (PMA) and ionomycin [235]. Treatment with PMA and ionomycin is sufficient to induce activation of many cell types to produce cytokines [236, 257]. This cocktail can be used to induce cytokine production in *in vitro* cell culture for subsequent detection in immunoassays, either by ELISA, cytometric bead array [258], or intracellular cytokine identification [155]. PMA is a small organic compound which diffuses through the cell membrane into the cytoplasm, where it directly activates protein kinase C (PKC). Activation of PKC triggers calcium ion release and mobilisation, resulting in a cascade of additional cellular responses mediating T-cell activation [259]. Ionomycin, a calcium ionophore produced by the bacterium *Streptomyces conglobatus* , is used in addition to PMA to trigger calcium release, which is required for nuclear factor of activated T-cell (NFAT) signalling [260]. Stimulation with these compounds bypasses the TCR complex and will lead to activation of several intracellular signalling pathways [261]. This activation results in a rapid up-regulation of cytokine production.

Previously, the combined stimulation of PMA and ionomycin was used in analysis of intracellular cytokines following a 2.5 h bout of cycling [155, 159], and a 1.5 h bout of running [159]. Both studies demonstrated exercise-induced changes in type 1 cytokine-producing T cells as described in Chapter 1.4.5.

1.8 Exercise Physiology

It is necessary to consider a number of physiological variables related to exercise when examining immune changes due to exercise-induced stress. As outlined above, stress hormone release in dictated by both exercise intensity and duration. As such, when designing a study to examine the influence of a set exercise intensity, it is paramount to understand the physiological effects of exercise of varying intensity on the stress response. Below are a number of variables important to consider when prescribing a bout of exercise.

1.8.1 Oxygen Consumption

Oxygen consumption/uptake is a measure of metabolic demand. Changes in oxygen consumption are driven by changes in underlying metabolic processes, as the consumption of oxygen is necessary for sustain production of adenosine triphosphate (ATP) from substrates (e.g. glucose, free fatty acids) [131]. When exercise begins, the increase in energy production is used to provide ATP for contracting skeletal muscles. This ATP is provided by an increase in aerobic metabolism, driving greater consumption of oxygen. This oxygen consumption can be measured by open-circuit spirometry to accurately determine resting metabolic rate, and changes in oxygen consumption and carbon dioxide production during rest and exercise [262].

1.8.2 $\dot{V}O_2$max/$\dot{V}O_2$peak

Maximal oxygen uptake is an important measure for when quantifying exercise intensity. It is defined as the highest oxygen uptake that an individual can obtain during exercise using large muscle groups [263]. The maximal capacity to transport and utilise oxygen during exercise is considered by many to be the most valid measurement of cardiovascular fitness and has a high correlation with aerobic performance [132]. The most accurate means to determine $\dot{V}O_2$max is by direct laboratory measurement. This is generally performed in the form of an incremental (or graded) exercise test in a laboratory using a motorised treadmill or cycle ergometer, and open-circuit spirometry used to measure pulmonary gas-exchange. The terms peak oxygen uptake ($\dot{V}O_2$peak) and maximal oxygen uptake ($\dot{V}O_2$max) are both used in this thesis. The difference in terms is dictated by whether the subject reaches

specific criteria for maximum oxygen consumption. The primary criterion for reaching $\dot{V}O_2$max is a plateau in $\dot{V}O_2$ (<200 mL.min^{-1} change) with increasing work rate. Additionally, secondary criteria for reaching $\dot{V}O_2$max include; 1) a maximal heart rate within 5% of their age predicted maximal heart rate (220 beats.min^{-1} - age), 2) a blood lactate concentration of >11.0 mmol/L collected 5 min post-exercise, and 3) respiratory exchange ratio of ≥1.10 [264]. If these criteria are not met, then the highest oxygen consumption result would be defined as $\dot{V}O_2$peak.

1.8.3 LT/AT/VT_1

An even more accurate measure of exercise intensity is by comparing workload in relation to the lactate threshold (LT) – also known as the anaerobic threshold (AT), or the first ventilatory threshold (VT_1). It is thought that below the lactate threshold, ATP production used to provide energy for muscle contraction comes from aerobic sources [262, 265]. However, as exercise intensity increases, so does reliance on anaerobic metabolism [266-268]. As such, an exponential rise in blood lactate concentration occurs, defined as the LT. Depending on the method of identification, the point at which this occurs can be described as the LT, AT, or VT_1. While, LT is defined by analysis of blood lactate concentrations during an incremental exercise test, AT and VT_1 are identified using respiratory exchange data from the same protocol. As blood lactate concentration, blood pH, CO_2 production, and ventilation ($\dot{V}_E$) are closely related, gas exchange data can be used to non-invasively identify these points [269]. To determine AT, the relationship between $\dot{V}O_2$ and $\dot{V}CO_2$ is examined by regression analysis, called the V-slope method [270]. The highest work rate prior to the systematic departure from a linear relationship between $\dot{V}O_2$ and $\dot{V}CO_2$ is defined as AT, which occurs secondary to an excess CO_2 production from non-metabolic sources, thus increasing $\dot{V}CO_2$ [270, 271]. To determine VT_1, the ventilatory equivalents for O_2 ($\dot{V}_E/\dot{V}O_2$) and CO_2 ($\dot{V}_E/\dot{V}CO_2$) are plotted against work rate. VT_1 is then defined as the exercise intensity at which $\dot{V}_E/\dot{V}O_2$ exhibited a systematic increase without a concomitant increase in $\dot{V}_E/\dot{V}CO_2$.

1.8.4 MLSS/RCT/VT_2

Another term commonly used in relation to changes in lactate concentration with increasing exercise intensity is the "maximal lactate steady state" (MLSS). This is defined as the highest exercise intensity at which blood lactate concentration, while elevated compared with rest, may attain a steady-state value and remains at equilibrium for the remainder of the exercise [272]. Teamed with this increase in blood lactate are a number of changes in $\dot{V}_E$ and $\dot{V}CO_2$, allowing this point to be identified by gas exchange data [273-275]. As with the thresholds above, the respiratory compensation threshold (RCT) and second ventilatory threshold (VT_2) can be determined by analysis of $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$. This point corresponds to the exercise intensity at which an increase in $\dot{V}_E/\dot{V}O2$ is accompanied by an increase of $\dot{V}_E/\dot{V}CO2$ [267]. This point can also be defined as the work rate associated with the second non-linear increase of $\dot{V}_E$ and $\dot{V}CO2$ [270].

1.8.5 Exercise Intensity Domains

Exercise intensity domains are used to define the points between different thresholds. This provides insight into exercise intensity, and how sustainable such a bout of exercise would be. These domains can be divided into "moderate", "heavy", "severe", and "extreme" [276]. Any exercise intensity correspond to an oxygen uptake between rest and that corresponding to LT/AT/VT_1 is defined as the moderate intensity domain. Exercise intensities above LT/AT/VT_1 but less than MLSS/RCT/VT_2 are defined within the heavy-intensity domain. Any exercise intensity performed above MLSS/RCT/VT_2 and less than $\dot{V}O_2$max is defined as the severe intensity domain. Finally, extreme-intensity is above MLSS but $\dot{V}O_2$peak is not achieved as fatigue occurs before $\dot{V}O_2$ reaches asymptote [276]. As a guide, both the moderate and heavy-intensity domains are sustainable for a prolonged time period, whereas exercising in the severe domain would result in an upward drift of $\dot{V}O2$ towards maximal values, at which point, exhaustion would occur.

1.8.6 Prolonged, Heavy-Intensity Exercise

As this term is mentioned throughout the current thesis, it is necessary to define "prolonged heavy-intensity exercise". For the purpose of the following studies, prolonged heavy-

intensity exercise is defined as sustainable exercise of duration greater than 1.5 h, at work rates above the anaerobic threshold. Previous research has highlighted a significant increase in training volume (both; proportion (%), and absolute volume (km)) in the heavy-intensity domain when transitioning from the off-season to pre-competition, and competition phases of a season in professional cyclists [20]. Sustainable power at the upper limit of the heavy-intensity domain is correlated to time trial performance [277]. Furthermore, during “le Tour de France”, cyclists averaged ~60% of a climb’s duration (hors, first, and second category climbs) at or above the heavy-intensity domain [278]. Cyclists are regularly training and racing at this exercise intensity for prolonged periods of time, as such it was deemed appropriate that our studies mirror such an exercise dose.

1.9 Aims of the Current Study

The overall aim of this thesis is to analyse the immune system response and subsequent recovery period following prolonged heavy-intensity exercise in highly trained individuals under various conditions. More specifically:

- To analyse the effect of a single bout of prolonged heavy-intensity exercise on a number of novel markers of immune system function including, dendritic cells, γδ T cells, and T helper cytokines. Focus will be placed both on the immediate exercise effect and the subsequent immune recovery period after exercise.
- To determine the effect of prior exercise on the immune system response and recovery period following a second bout of exercise with minimal recovery between sessions, by examining a number of novel markers of the immune system.

Below are the specific aims and hypotheses for Studies I, II, and III.

The aim of Study I is to examine the effect of prolonged heavy exercise on plasma cortisol concentration, and Th1, Th2, and Th17 cytokine release following exercise and recovery. We hypothesised that the exercise-induced stress would increase plasma cortisol concentration after exercise, resulting in the release of IL-17 and a shift from secretion of Th1 to Th2 cytokines, returning to resting level several hours after the cessation of exercise.

The aim of Study II is to examine the effect of prolonged heavy exercise on circulating concentrations of lymphocyte and dendritic cells and their subsets following exercise and recovery. We hypothesised that the exercise-induced stress would increase circulating concentrations of cell subsets following exercise, with those cell subsets presenting more effector characteristics highlighting ingress into circulation at a higher degree than those cell subsets with more naïve cell functions, returning to resting level several hours after the cessation of exercise.

The aims of Study IIIA and Study IIIB are to examine the effect of repeated, prolonged heavy exercise on Th1, Th2, and Th17 cytokine release, in addition to lymphocyte and dendritic cells and their subsets following exercise and recovery. We hypothesised that repeated exercise would result in a ‘carry on’ or additive effect compared to a single bout

of exercise. This would manifest itself in a greater peak in cell concentrations post-exercise, a greater release of IL-17 and a more pronounced shift from secretion of Th1 to Th2 cytokines.

1.10 Significance

The multifaceted nature of the immune system, and the varying influence of exercise and stress on the immune function, there is a great deal that is unknown in the field of exercise immunology. Fortunately, this leaves many niches of the immune system unexplored in its response to many forms of exercise and stress. Because of variability between studies in training status of the subjects, exercise protocols, analysis, and sampling, many areas can be explored. The immune system represents a hugely complex, communicating, fine-tuning system in which the stress response has large influence over its function. More research is warranted to explore the relationships between stress, exercise, and immune function. In the present study, this involves examining rarely tested markers (such as γδ T cells , dendritic cells and T helper cytokines), under controlled conditions, and circumstances that may be expected to produce greater, potentially more harmful effects on the immune system, both during the stress, and during the subsequent recovery period.

The exercise protocols employed for the present studies have been designed to mirror the heavy training intensity and duration experienced by elite cyclists. The results may provide a more complete understanding of the effects heavy prolonged exercise have on the immune system in athletes who include repeat training sessions in their regimes. The results of this study may help identify contributing factors accounting for the illness that some athletes experience during phases of intense training combined with short recovery periods. Additionally, it is hoped this research may help identify when the immune system has recovered, potentially indicating when it is safe – in an immune health context – for an athlete to return to training.

2. METHODOLOGY

2.1 Study Design

Three studies were proposed to investigate cellular and cytokine changes in response prolonged heavy-intensity exercise. A large focus was placed on the temporal characteristics and kinetics during the recovery period of the dependant variables. Analysis included a number of immune system variables, and the inclusion of both single and repeated exercise protocols.

2.1.1 Study I

Study I aimed to examine the effect of a single two-hour bout of prolonged heavy-intensity exercise on stimulated Th1/Th2/Th17 cytokine concentrations in peripheral blood. The post-exercise recovery period was of key interest, featuring six sampling points after the completion of exercise.

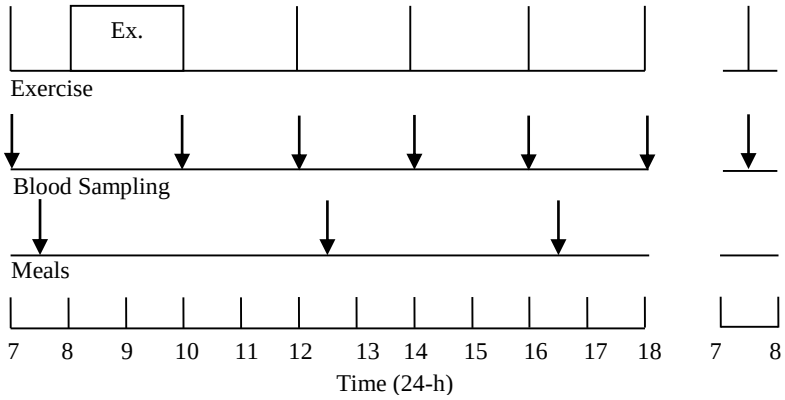

Figure 2.1. Time-line of blood sampling and meal schedule Study I. Time points include pre-exercise, immediately post-exercise, 2 h, 4 h, 6 h, 8 h, and 24 h post-exercise.

2.1.2 Study II

The aim of Study II was to examine the effects a single two-hour bout of prolonged heavy-intensity exercise on total dendritic cell concentrations and dendritic cell subsets, and γδ T cell concentrations, γδ T cell phenotypes in peripheral blood. The focus was to analyse the immediate effect of the exercise and the subsequent changes during the post-exercise period.

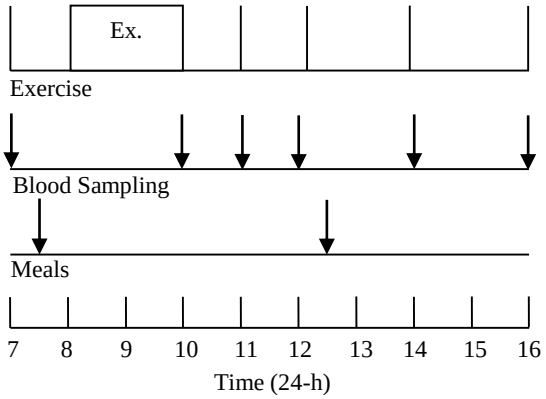

Figure 2.2. Time-line of blood sampling and meal schedule Study II. Time points include pre-exercise, immediately post-exercise, 1 h, 2 h, 4 h, and 6 h post-exercise.

2.1.3 Study III

Study III aimed to examine the effect of repeated ninety-minute exercise bouts on perturbations in the immune system following prolonged heavy-intensity endurance exercise. Analyses included full blood count (FBC), stimulated Th1/Th2/Th17 cytokine concentrations, total dendritic cell concentrations and dendritic cell subsets, and γδ T cell concentrations, γδ T cell phenotypes in peripheral blood.

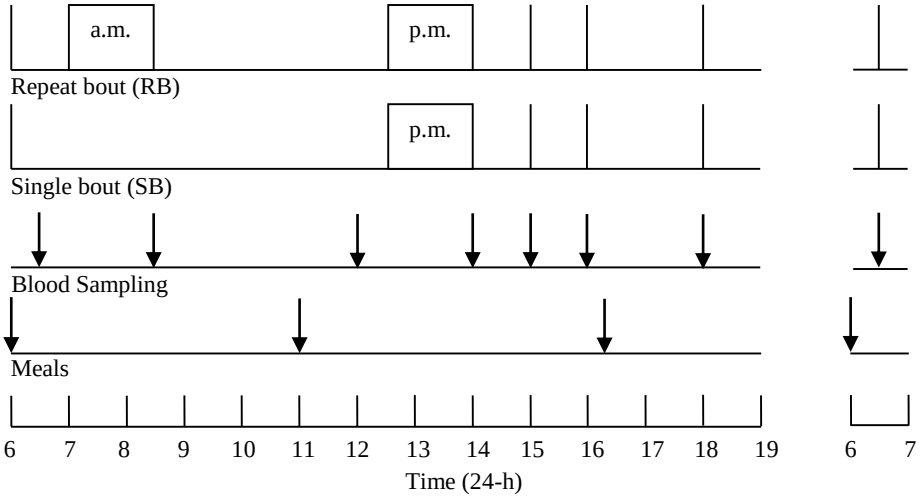

Figure 2.3. Exercise, blood collection, and meal schedule for the Single and Repeat bout data collection protocols (Study III).

2.2 Ethics

Ethical clearance for each study was obtained from the Bond University Human Research Ethics Committee (RO-955/RO-1145). Ethical approval of Study I (RO-955) was originally applied for in 2009. A number of amendments were added to RO-955 in 2011 to allow Study II to proceed. Finally, Study III ethical approval was obtained in RO-1145.

2.3 Subjects

Ten highly trained 'A' grade club-level male cyclists were recruited from various cycling clubs in South-East Queensland with the assistance of Cycling Australia for each study (total n = 30).

2.3.1 Power Calculations

Power calculations to predict recommended subject numbers were completed for repeated measures ANOVA. Effect size was calculated from reported *F*-statistics and degrees of freedom (*df*) published by Anane et al., 2009 and Anane et al., 2010 examining lymphocytes and specific lymphocyte subsets in response to short duration psychological stress task or a 16-min cycle bout at 85%W_{max} [104, 163]. Effect size was calculated online using the website; lyonsmorris.com/ma1/. *F* statistic, between groups *df*, within groups *df*, and the number of subjects were entered. Effect size was calculated for the following variables:

Table 2.1. Calculated effect size of psychological stress and acute exercise

Immune Variable	Psychological Stress	Acute Exercise
Lymphocyte concentration	0.3652	0.4296
NK cell concentration	0.3893	0.4317
γδ T cell concentration	0.3091	0.3803
δ1 T cell concentration	0.2902	
δ2 T cell concentration	0.3024	

Sample size was calculated using G*Power software for ANOVA with repeated measures, within-factors interaction, for Study I and Study II. For the above variables, statistical power was set at 0.8, alpha set at 0.05, sample size was calculated as follows:

Table 2.2. Calculated sample size from effect size calculations for Study I and Study II

Immune Variable	Psychological Stress	Acute Exercise
Lymphocyte concentration	8	6
NK cell concentration	7	6
γδ T cell concentration	11	8
δ1 T cell concentration	12	
δ2 T cell concentration	11	

Sample size was calculated using G*Power software for ANOVA with repeated measures, within-between interaction, for Study III. For the above variables, statistical power was set at 0.8, alpha set at 0.05, sample size was calculated as follows:

Table 2.3. Calculated sample size from effect size calculations for Study III

Immune Variable	Psychological Stress	Acute Exercise
Lymphocyte concentration	6	6
NK cell concentration	6	6
γδ T cell concentration	8	6
δ1 T cell concentration	8	
δ2 T cell concentration	8	

When using the effect size of previous research, it should be noted that these studies used short-duration, acute psychological stress and a 16-minute cycle bout at 85%W_{max}. Compared to prolonged, heavy-intensity exercise of this proposed thesis, the interventions used by Anane et al., would likely elicit a response of reduced magnitude compared to prolonged exercise. This can be seen when comparing the acute psychological stress to the

16-minute cycle bout at 85%W_{max}. Additionally, previous research in exercise immunology have indicated groups of 9 – 10 subjects are adequate to demonstrate significant changes in immune variables including cortisol concentration, lymphocyte concentration (including subsets $CD4^+$ T cells, $CD8^+$ T cells, NK cells), neutrophil concentration, and T helper cytokine concentrations [155, 177-179, 187, 189, 195-197, 279-282]. Based upon the results from these previous studies and the results from the power calculations above, a subject population of 10 subjects were recruited for each study.

2.4 Subject Screening

A pre-participation health screening was performed to ensure volunteers were able to undertake a maximal exercise test and prolonged heavy-intensity exercise. The Explanatory Statements and Consent Forms for Studies I, II, and III are located in the appendices (Appendix C to Appendix F). Potential participants completed a medical history questionnaire (Appendix G), exercise history questionnaire (Appendix H), and physical activity readiness questionnaire - PARQ (Appendix I). Further screening tests included; a pulmonary function test, a resting electrocardiogram (ECG) and blood pressure measurements, a finger prick blood test for blood glucose, total cholesterol, LDL cholesterol, and HDL cholesterol concentrations. The combination of the screening questionnaires and tests were used to detect potential pulmonary, cardiovascular, metabolic and immunological conditions. Data from the subject screening are presented in Tables 2.1, 2.2, and 2.3.

Table 2.4. Physical characteristics data collected during pre-screening for Study I

Subject Characteristics (n=10)	Mean ± SD
Age (years)	24.2 ± 5.3
Height (m)	1.82 ± 0.07
Body mass (kg)	73.8 ± 6.5
BMI ($kg.m^{-2}$)	22.2 ± 1.3
FVC (L)	5.98 ± 0.49
FEV_1 (L)	4.85 ± 0.40
FEV_1/FVC (%)	81.2 ± 2.4
Supine HR ($beats.min^{-1}$)	48.5 ± 11.2
Supine BP (mm Hg)	120.4 / 77.3 ± 5.9 / 4.8
Training (km/week)	292.5 ± 89.8

Data are mean ± standard deviation. BMI: body mass index. FVC: forced vital capacity. FEV_1: volume exhaled at end of first second of forced expiration. HR: heart rate. BP: blood pressure. Training: volume in kilometres performed during training per week.

Table 2.5. Physical characteristics data collected during pre-screening for Study II

Subject Characteristics (n=10)	Mean ± SD
Age (years)	24.0 ± 4.1
Height (m)	1.81 ± 0.05
Body mass (kg)	73.3 ± 5.8
BMI ($kg.m^{-2}$)	22.3 ± 1.4
Fasting glucose ($mmol.L^{-1}$)	4.4 ± 0.6
HDL ($mmol.L^{-1}$)	1.26 ± 0.32
LDL ($mmol.L^{-1}$)	2.90 ± 0.94
Total cholesterol ($mmol.L^{-1}$)	3.77 ± 1.06
HDL/Cholesterol	0.35 ± 0.1
FVC (L)	6.08 ± 0.62
FEV_1 (L)	4.90 ± 0.31
FEV_1/FVC (%)	81.1 ± 5.1
Supine HR ($beats.min^{-1}$)	46.0 ± 6.2
Supine BP (mm Hg)	119.2 / 73.6 ± 7.3 / 5.4
Training (km/week)	348 ± 57

Data are mean ± standard deviation. BMI: body mass index. HDL: high-density lipoprotein. LDL: low-density lipoprotein. FVC: forced vital capacity. FEV_1: volume exhaled at end of first second of forced expiration. HR: heart rate. BP: blood pressure. Training: volume in kilometres performed during training per week.

Table 2.6. Physical characteristics data collected during pre-screening for Study III

Subject Characteristics (n=10)	Mean ± SD
Age (years)	23.2 ± 3.2
Height (m)	1.82 ± 0.06
Body mass (kg)	74.8 ± 6.2
BMI ($kg.m^{-2}$)	22.5 ± 1.5
Fasting glucose ($mmol.L^{-1}$)	4.2 ± 0.8
HDL ($mmol.L^{-1}$)	1.46 ± 0.57
LDL ($mmol.L^{-1}$)	2.83 ± 1.08
Total cholesterol ($mmol.L^{-1}$)	3.64 ± 0.70
HDL/Cholesterol	0.39 ± 0.11
FVC (L)	5.74 ± 0.30
FEV_1 (L)	4.72 ± 0.56
FEV_1/FVC (%)	82.3 ± 7.8
Supine HR ($beats.min^{-1}$)	48.5 ± 11.2
Supine BP (mm Hg)	120.4 / 77.3 ± 5.9 / 4.8
Training (km/week)	316 ± 75

Data are mean ± standard deviation. BMI: body mass index. HDL: high-density lipoprotein. LDL: low-density lipoprotein. FVC: forced vital capacity. FEV_1: volume exhaled at end of first second of forced expiration. HR: heart rate. BP: blood pressure. Training: volume in kilometres performed during training per week.

A risk stratification matrix was employed to determine a potential participant's suitability for exercise [283]. This matrix examined positive risk factors including: age (≥ 45 years), family history (myocardial infarction, coronary revascularisation, or sudden death, in parents or other first degree relatives), cigarette smoking (current cigarette smoker, or those who quit within the previous 6 months), sedentary lifestyle (at least 30 minutes of moderate intensity physical activity, on at least three days of the week, for at least three months), obesity (body mass index ≥ 30 $kg.m^{-2}$ or waist girth > 102 cm), hypertension (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg), dyslipidemia (LDL cholesterol ≥ 3.37 $mmol.L^{-1}$, or HDL cholesterol < 1.04 $mmol.L^{-1}$, or total cholesterol ≥ 5.18 $mmol.L^{-1}$), and pre-diabetes (fasting plasma glucose ≥ 5.5 $mmol.L^{-1}$), and negative risk factor of high HDL cholesterol (≥ 1.55 $mmol.L^{-1}$). If a potential participant exhibited two or more risk factors, they were excluded from participation in the

research. Additional exclusion criteria included; 1) any contraindications to exercise (such as recent injuries and/or overt contraindicators for performing maximal exercise), 2) thyroid and/or other autoimmune diseases, and 3) immune deficiencies. Finally, potential participants were required to; 1) be between the ages of 18-35 years, 2) having a minimum of one year racing experience, and 3) currently racing in A Grade club racing or Elite A Open Grading.

2.5 Test Sessions

Subjects visited the exercise laboratory for a screening and familiarisation session, an incremental exercise test, and the experimental trial or trials for each study. An overview of the protocols used during these sessions is provided below.

2.5.1 Screening and Familiarisation Session

Once the screening process was completed (as described above), the participants underwent a familiarisation with the incremental exercise test protocol. This involved preparation of the cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) with assistance from the subject to determine their preferred bicycle setup. Ten electrodes were attached to each participant to examine the 12-lead ECG and record cardiac rate and rhythm, and a mouth-piece and pneumotachograph was positioned in the subject mouth to record gas exchange analysis (Ultima CardiO2, Medical Graphics Corp., St Paul, MN). The subjects then underwent the incremental exercise test protocol (including 4-min warm-up at 90 W, increasing 15 W every 30-sec thereafter) until they reached a heart rate of 120 beats.min^{-1}, at which point the test was terminated. Subjects were monitored for cardiac arrhythmias and abnormalities via ECG, and any further contraindications to exercise and signs of poor perfusion such as injury, confusion, ataxia, pallor, cyanosis, nausea, or cold and clammy skin. Subjects were able to stop the test at any time. During this session, preferred cycling cadence was determined by the subject and researchers, which was subsequently used during incremental exercise test and experimental trials. All trials (screening and familiarisation, incremental exercise test, and experimental trials) were performed in a laboratory with the temperature controlled at 20-22°C.

2.5.2 Incremental Exercise Test

The incremental exercise test was used to determine each subject's individual ventilatory thresholds and $\dot{V}O_2peak$. Following completion of the pre-screening and familiarisation process (as described above), subjects undertook the incremental exercise test. Immediately before the test, the metabolic cart was calibrated according to the manufacturer's instructions. The pressure-differential pneumotachograph was calibrated before each test

by using a syringe of known volume (3.0 L) over a range of flows. Oxygen and carbon dioxide gases were then calibrated before each test using a precision-analysed gas mixture. The cycle ergometer was prepared according to the previously determined preferred cycling position to ensure consistency between all exercise bouts. Once the subjects were prepared with the 12-lead ECG, mouth-piece and nose clip, they were invited to sit on the bike. The incremental exercise test involved a 4-min warm-up at 90 W. Following the warm-up the resistance applied on the cycle ergometer increased at a rate of 15 W every 30-sec. Subjects were required to remain at the same cadence throughout the test. Data collected included cardiac rate and rhythm, blood pressure and gas exchange data. Gas exchange data was collected breath-by-breath, with 30-sec and 60-sec averages recorded post-hoc. Criteria for termination of the test included any heart abnormalities, faintness, and any further contraindications to exercise as listed above. Furthermore, subjects were permitted to end the test at any time for any reason. If the participant's cadence decreased 15 revolutions.min^{-1} below the predetermined preferred cadence, despite verbal encouragement, the test was terminated. Each subject's individual $\dot{V}O_2$peak was determined by their highest $\dot{V}O_2$ value from 30-sec averaged $\dot{V}O_2$ data. Criteria for reaching $\dot{V}O_2$max included: 1) a plateau in $\dot{V}O_2$ (<200 mL.min^{-1} change) with increasing work rate, 2) a maximal heart rate within 5% of their age predicted maximal heart rate (220 beats.min^{-1} - age), and 3) respiratory exchange ratio of ≥1.10 in which any two of the above three criteria indicate reaching $\dot{V}O_2$max [264].

Threshold Determination

Each subject's ventilatory thresholds (VT_1/VT_2) were determined by their gas-exchange data collected during the incremental exercise test using the following criteria. The first ventilatory threshold (VT_1) was defined as the exercise intensity at which $\dot{V}_E/\dot{V}O2$ exhibited a systematic increase without a concomitant increase in $\dot{V}_E/\dot{V}CO2$. The second ventilatory threshold (VT_2) corresponded to the exercise intensity at which the increase in $\dot{V}_E/\dot{V}O2$ was accompanied by an increase of $\dot{V}_E/\dot{V}CO2$ [267]. Thresholds were further defined as the work rates associated with a first and a second nonlinear increase of $\dot{V}_E$ and $\dot{V}CO2$ [270]. According to the criteria outlined above, two independent investigators

reviewed the plots of each index and made individual determinations of VT_1 and VT_2. If the investigators did not agree on a particular workload for VT_2 by two or more workload levels, a third investigator was consulted. If the investigators chosen workload for VT_2 were one workload apart, an average of the two was taken.

2.5.3 Experimental Trials

Subjects were asked to train normally in the lead up to the test sessions, however it was requested that the subjects refrained from high intensity exercise during the two days prior to each experimental trial and avoid exercise for 24 h prior to visiting the laboratory. Furthermore, subjects were asked to avoid the use of large-dose vitamin/mineral supplements (above 100% of recommended dietary allowances), nutritional supplements, ergogenic aids, herbs, and medications known to affect immune function for one week prior to the test sessions.

All exercise bouts were performed at the same intensity and duration for each subject, consisting of a 5-min warm-up period at 50% of $\dot{V}O_2$peak, immediately followed by either a 90-min (Study III) or 120-min (Study I and Study II) cycling bout at the predetermined workload (90% of the each individual's second ventilatory threshold) at the subject's previously determined preferred cadence. Gas exchange was measured for 4 min at 20-min intervals during the exercise bout to ensure the workload remained consistent. Once the exercise trial was complete, subjects were asked to sit while blood samples were taken.

The subjects were under supervision for the entirety of the sampling, except overnight. While under supervision, subjects were given the option to read, listen to music, watch TV or work on their computer. Subjects were not permitted to sleep during the day of the experimental trial.

Meal Protocol

Total energy requirements per subject were calculated for the day of the experimental trial. These total requirements were sub-divided into main meals plus snacks at specific times (Figure 2.1, Figure 2.2, Figure 2.3). As used in previous studies [195, 196], to ensure

adequate carbohydrate intake, all meals consisted of a minimum of 60% carbohydrates. While under supervision, all meals were provided.

2.5.4 Blood Sample Collection

A qualified phlebotomist was employed to draw blood at each time point. Blood was drawn from an antecubital vein by venepuncture or cannulation. Blood collection points varied between studies, as previously described above (Figure 2.1, Figure 2.2, and Figure 2.3).

2.6 Flow Cytometry

2.6.1 Th1/Th2/Th17 Cytokines

Th1/Th2/Th17 cytokines were assessed using the Human Th1/Th2/Th17 Cytokine Kit (Becton Dickson (BD) Bioscience, California, USA). The test kit quantitatively measured the concentration of selected circulating cytokines in the blood [229, 284]. It contained antibodies specific to IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A proteins. The cytometric bead array system was employed to assess and quantitatively measure the concentration of plasma cytokines from peripheral blood mononuclear cells (PBMCs) and whole blood. Two different mitogens and stimulation methods were employed. Stimulation of PBMCs by PHA was used in Study I, whereas stimulation of whole blood by PMA and ionomycin was used for Study III. The decision to change mitogen was reached before Study III, as the results from Study I proved difficult to interpret. It was deemed a more broad-spectrum approach via PMA-stimulation of whole blood to the examine T helper cytokines may provide a response more indicative of immune function compared to the more narrow view provided by isolated PBMC analysis. The large difference in incubation time described in detail below; 72 h with PHA and 4 h with PMA and ionomycin, is due to the severity in which these individual mitogens act. Previous research has suggested a range of 48 to 72 h for PHA stimulation and 4 to 6 h for PMA [235, 236, 241, 243, 285-287].

The protocol for Study I involved the following; 10 mL of blood was collected into lithium heparin tubes (BD Bioscience) and layered over Ficoll-Paque PLUS (GE Healthcare Life Sciences, Milan, Italy). Following centrifugation at 400 x g for 40 min, cells were resuspended in RPMI-1640 culture medium containing 5% foetal bovine serum (FBS) and then counted (Countess® Automated Cell Counter, Life Technologies, Carlsbad, CA). Isolated PBMCs were mitogenically stimulated with or without 1 µg of phytohaemagglutinin (PHA) at a concentration of $1x10^6$ cells/mL at 37°C with 5% CO_2 for 72 hours. Following incubation, the supernatant was removed and stored at −80°C for subsequent analysis.

The protocol employed for Study III differed to Study I. Briefly, 5 mL of blood was collected into lithium heparin tubes. Two tubes were prepared per sample, each containing

a volume of 1 mL of whole blood and 1 mL of RPMI medium. To the stimulated sample, PMA (25 ng/mL) and ionomycin (1 µg/mL) were added, whereas nothing more was added to the unstimulated sample. All samples were mixed and then incubated for 4 h at 37°C. Following incubation, the supernatant was removed and stored at −80°C for subsequent analysis.

Table 2.7. Lower limits of detection of Th1, Th2, and Th17 cytokines

Cytokine	Limit of detection (pg/mL)
IL-2	2.6
IL-4	4.9
IL-6	2.4
IL-10	4.5
TNF	3.8
IFN-γ	3.7
IL-17A	18.9

Th1, Th2 and Th17 cytokine concentrations were quantified using a commercially available cytometric bead array kit (BD Pharmingen, San Diego, CA) to measure the concentrations of IL-2, IL-4, IL-6, IL-10, TNF, INF-γ and IL-17A from supernatant of stimulated samples. In the absence of stimulation with PHA or PMA and ionomycin, the concentrations of these cytokines were not within the limits of detection. Limits of detection are included in Table 2.4. The range of standards for each cytokine were prepared as follows; 0 pg/mL, 20 pg/mL, 40 pg/mL, 80 pg/mL, 156 pg/mL, 312.5 pg/mL, 625 pg/mL, 1250 pg/mL, 2500 pg/mL, and 5000 pg/mL. Samples were diluted as a ratio of 1:4 with assay diluent. Next, 50 µL of each unknown sample was mixed with 50 µL of Human Th1/Th2/Th17 PE detection reagent and allowed to incubate for 3 hours. Following incubation, samples were washed with wash buffer, and then resuspended for acquisition.

Prior to sample analysis, the BD CBA Dual-Laser Instrument Setup template for acquisition of cytokines using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine kit were downloaded from bdbioscience.com/cbatemplates and installed onto the flow cytometer. Instructions for setup of the correct acquisition template and instrument settings were available at the website;

bdbioscience.com/documents/CBA_HumanTh1Th2Th17_Kit_Manual.pdf. Briefly, cytometer setup beads were prepared with the inclusion of FITC and PE Positive Controls. SSC, FSC, FL1, and FL2 PMT voltages were optimised to gate the desired bead population.

Acquisition of samples was set at 2100 gated events as per the kit instructions. All standards were acquired, followed by the unknown samples. Analysis of Human Th1/Th2/Th17 Cytokine data was completed using FCAP Array software. Instructions for analysis were available at; bdbioscience.com/docs/FCAP_Array_analysis_of_CBA_Kits.pdf. Briefly, bead groups were defined for each cytokine analysed. Next, standards, unknown samples, and the dilution factor were defined. Finally, standard curves were produced and unknown sample concentrations were calculated.

2.6.2 γδ T cell subset

Total γδ T cells, δ1 and δ2 subsets, memory phenotypes, and surface molecules were determined using the following antibodies; CD3 PerCP (BD Biosciences), Vδ-1 TCR FITC (Thermo Scientific), γδ TCR PE, Vδ-2 TCR APC, CD27 FITC, CD27 PE, CD45RA APC, CD11a FITC, CD62L PE, CD94 FITC (BD Pharmingen) [163]. Briefly, 100 µL whole blood was incubated with appropriate amounts of antibody (as determined by titration, Table 2.5) for 30 min in the dark at room temperature. Volumes used, and tube arrangement of each antibody used are included in Table 2.5. Erythrocytes were subsequently lysed using FACS lysing solution (BD Bioscience). Following lysis, samples were centrifuged (350 × *g*, 5 min, at room temperature). Samples were then washed and fixed in 1% paraformaldehyde. Fixed preparations were analysed immediately, collecting a minimum of 100,000 events from each sample.

Appropriate, matched isotype controls were used to set negative staining criteria. Fluorescence-minus-one (FMO) controls were not required to asses spill-over as four colour flow cytometry was being used [228, 288]. The FACS-Calibur flow cytometer (BD Biosciences) was calibrated weekly using Calibrite beads (BD Biosciences). Total

lymphocytes were gated using the forward versus side scatter plot to identify the target population. γδ T cells were then gated as the $CD3^+$ γδ TCR^+ population, δ1 T cell were gated as $CD3^+V\delta1^+$ and δ2 T cells were gated as $CD3^+$ $V\delta2^+$. Additional information on the gating strategy is presented in Appendix A. Lymphocyte subset concentrations were quantified using the percentage values obtained from each lymphocyte subset gate. The total lymphocyte concentration was obtained from the full blood count.

Table 2.8. γδ T cell assay antibody volumes and tube assignment

Antibody	Fluorochrome	Volume (µL)	Tube
CD3	PerCP	10	A, B, C, D
Vδ-1 TCR	FITC	5	A
Vδ-2 TCR	PE	5	B
γδ TCR	APC	5	C, D
CD27	FITC	10	B
CD27	PE	10	A
CD45RA	APC	10	A, B
CD11a	FITC	10	C
CD62L	PE	10	C
CD94	FITC	10	D

2.6.3 Dendritic cell phenotypes

Total dendritic cells (tDCs), dendritic cell phenotypes (pDCs, mDCs, $CD14^{-/low}CD16^+$ DCs), and total monocytes were identified by the combination of HLA-DR PerCP, lineage cocktail-2 FITC (CD3, CD14, CD19, CD20, CD56), CD123 PE (BD Bioscience), CD33 APC, CD11c PE and CD16 APC (BD Pharmingen) antibodies. Briefly, 100 µL whole blood was incubated with appropriate amounts of antibody (as determined by titration, Table 2.6) for 30 min in the dark at room temperature. Volumes used, and tube arrangement of each antibody used are included in Table 2.6.

Expression of CD33 and HLA-DR and absence of the lineage cocktail-2 antibodies with intermediate forward and side scatter characteristics located between those of lymphocytes

and monocytes was used to determine tDCs. Dendritic cells were further defined into three subpopulations: (1) a subset of $CD123^{dim}/CD33^{high}/CD16^{-}$ cells, with strong expression of HLA-DR–mDCs; (2) a subset of $CD123^{inter}/CD33^{inter}/CD16^{+}$ cells–$CD14^{-/low}CD16^{+}$ DCs and (3) $CD123^{high}/CD33^{-/dim}/CD16^{-}$ cells–pDCs, both with intermediate expression of HLA-DR [289]. Additional information on the gating strategy is presented in Appendix B. Cell concentrations were quantified using percentage values obtained from each lymphocyte and monocyte subset gate. The total monocyte concentration was obtained from the full blood count.

Table 2.9. Dendritic cell assay antibody volumes and tube assignment

Antibody	Fluorochrome	Volume (µL)	Tube
HLA.DR	PerCP	10	A, B
Lineage cocktail-2 (CD3, CD14, CD19, CD20, CD56)	FITC	10	A, B
CD33	APC	10	A
CD123	PE	10	A
CD16	APC	10	B
CD11c	PE	10	B

2.7 Additional Analysis

2.7.1 Plasma Cortisol

Samples for cortisol analysis were collected in EDTA anti-coagulation blood collection tubes. Samples were spun at 5000 x g for 10 min. Plasma was removed and stored at -80°C until analysis. Analysis was completed as previously described [290]. Samples were analysed by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC MS/MS) described elsewhere [290]. Briefly, plasma samples were treated with 1 M HCl at room temperature to displace cortisol and cortisone from binding proteins. To each sample, the multi-analyte internal standard (d4-dexamethasone, d2-11-deoxycortisol and d2-cortisone) and H_2O were added before loading onto activated Oasis HLB 1 cc (30 mg) solid phase extraction cartridges (Waters, Milford, USA). Samples were eluted with 100 % ethyl acetate, dried at 50°C and reconstituted in 45 % methanol with 2 mmol/L ammonium acetate and 0.1 % formic acid. The flow rate of the UHPLC analysis was 0.4 ml/min, sample injection volume 20 µl, and column temperature 50°C. The tandem mass spectrometer was operated in positive mode electron spray ionisation. The total analytical run time on the UHPLC MS/MS was 3 min.

2.7.2 Full Blood Counts

Full blood counts were performed using a five-part differential haematology analyser (Beckman Coulter AcT 5 diff AL Hematology Analyzer, California, USA). The haematology analyser utilised a sequential dilution system and dual-focused flow fluid dynamics technologies employing the Coulter Principle of impedance to count and size the cells. Plasma volume calculations and adjustments were conducted for all studies according to Dill and Costill [291]. Data presented has been adjusted for plasma volumes shifts. No significant change in plasma volume was evident in any study.

2.8 Statistical Analysis

Due to the divergent statistical analyses employed for each study, the statistical methodology is described in detail in each individual chapter.

3. STUDY I – T HELPER CELL CYTOKINE PROFILES FOLLOWING ENDURANCE EXERCISE

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Key words: Exercise, Immunology, Cytokines, Helper T-Lymphocytes

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Abstract

Endurance exercise can cause immunosuppression and increase the risk of upper respiratory illness. The present study examined changes in the secretion of T helper (Th) cell cytokines following endurance exercise. Ten highly-trained road cyclists (mean ± SEM: age 24.2 ± 1.7 yr; height 1.82 ± 0.02 m; body mass 73.8 ± 2.0 kg; peak oxygen uptake 65.9 ± 2.3 $ml.kg^{-1}.min^{-1}$) performed 2 h of cycling exercise at 90 % of the second ventilatory threshold. Peripheral blood mononuclear cells were isolated, and stimulated with phytohaemagglutinin. Plasma cortisol concentrations and the concentration of Th1/Th2/Th17 cell cytokines were examined. Data were analysed using both traditional statistics and magnitude-based inferences. Results revealed a significant decrease in plasma cortisol at 4 h to 24 h post-exercise, compared to pre-exercise values. Qualitative analysis revealed post-exercise changes in concentrations of plasma cortisol, IL-2, TNF, IL-4, IL-6, IL-10, and IL-17A compared to pre-exercise values. A Th1/Th2 shift was evident immediately post-exercise. Furthermore, for multiple cytokines including; IL-2 and TNF (Th1), IL-6 and IL-10 (Th2), and IL-17 (Th17) no meaningful change in concentration occurred until more than 4 hours post-exercise, highlighting the duration of exercise-induced changes in immune function. These results demonstrate the importance of considering 'clinically' significant versus statistically significant changes in immune cell function following exercise.

Introduction

Prolonged endurance exercise is associated with increased incidence of symptoms associated with upper respiratory illnesses (URIs) in elite athletes [29, 31, 32, 292]. This increased rate of URIs may be observed following acute bouts of prolonged heavy- to severe-intensity exercise (e.g., marathon running, triathlon, road cycling) and periods of increased training load, potentially inducing a chronic stress response [108]. The aetiology of exercise-induced URIs appears to be multifactorial, as the associated stress response involves many different cell types, pathways and proteins of the immune system [111].

Stress-related changes in immune system function are typically mediated through activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis. Activation of the SNS induces catecholamine release, with a delayed release of glucocorticoids. These stress hormones alter immune function after acute exercise and during periods of heavy training [107], in response to acute and chronic psychological stress [293], and following surgery [294]. The effects of exercise-induced stress hormones on T helper cell function during and after prolonged heavy-intensity exercise has not been fully elucidated.

Glucocorticoids and catecholamines influence T helper cell (Th) cytokine release, principally by stimulating glucocorticoid and β_2 adrenergic receptors, respectively [166]. In turn, these stress hormones influence the activity of cellular (Th1), humoral (Th2), and mucosal (Th17) branches of the immune response [166, 167]. Specifically, cortisol, epinephrine and norepinephrine may suppress cytokine release from antigen presenting cells and Th1 cells, while up-regulating cytokine production from Th2 cells, thereby causing a shift toward Th2 cytokine production [155, 168]. The balance between Th1 and Th2 immunological responses is very important in maintaining optimum immune health. The Th1 pathway primarily acts against intracellular pathogens, particularly viruses and bacteria, while the Th2 pathway is believed to protect against extracellular pathogens such as parasites [101]. Previous research has extensively examined changes in the balance of Th1 and Th2 cytokines in plasma in the first 1–2 h after exercise [295]. By contrast, there is less research published concerning changes in T helper cytokine secretion (including

Th17 cytokines) during recovery from prolonged heavy exercise. While these changes are transient, it may have implications related to potential immune suppression following prolonged heavy-intensity exercise [296].

Exercise-induced glucocorticoid release occurs during prolonged exercise [107]. An increase in glucocorticoid concentration after exercise may influence T helper cytokine secretion differently to exercise of moderate duration and intensity, due to the large effect of changes in cortisol on immune function [107, 111]. Th17 cells play a key role in mucosal host defence of the upper respiratory and gastrointestinal tracts against a myriad of airborne antigens [297-299]. Functioning as part of the "first line of defence" in the mucosa, Th17 cytokines have been linked to increased polymeric immunoglobulin receptor (pIgR) expression, saliva sIgA and human β-defensin 2 release [300-303]. These results demonstrate that Th17 cytokine pathways are influential in the innate immune response in the mucosa. However, there is little evidence available about changes in Th17 cytokines after exercise.

The aim of the present study was to examine the effect of prolonged heavy exercise on plasma cortisol concentration, and Th1, Th2, and Th17 cytokine release. We hypothesised that the exercise-induced stress would increase plasma cortisol concentration after exercise, resulting in the release of IL-17 and a shift from secretion of Th1 to Th2 cytokines.

Methods

Subjects

Ten well-trained male cyclists volunteered to participate in the present study (mean ± SEM: age 24.2 ± 1.7 yr; height 1.82 ± 0.02 m; body mass 73.8 ± 2.0 kg; peak oxygen uptake 65.9 ± 2.3 $ml.kg^{-1}.min^{-1}$). Subjects were excluded if they exhibited any cardiovascular, metabolic, neurological, immunological or autoimmune disorders. The cyclists provided written informed consent for participation in the study, which was approved by the Bond University Human Research Ethics Committee.

Experimental Design

The cyclists visited the laboratory on three occasions; for a screening/familiarisation session, a maximal exercise test, and a constant load test with blood sampling before and after exercise. Peak oxygen consumption ($\dot{V}O_2peak$) was determined during a graded exercise test in which the cyclists were allowed to choose a preferred cadence within the range of 70–90 $rev.min^{-1}$. The test was performed on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The cyclists commenced cycling at 90 W for 4 min; thereafter intensity increased by 30 W/min until volitional fatigue and/or when cadence could not be maintained at a minimum 70 $rev.min^{-1}$. The criteria to determine maximal efforts at the end of a test were: a plateau in $\dot{V}O_2$ (defined as an increase in $\dot{V}O_2$ values of less than 1.5 $ml.kg^{-1}.min^{-1}$ between two consecutive 1-min periods in the final part of the test), a maximal value of respiratory exchange ratio ≥ 1.1, a maximal heart rate value above 95 % of the age-predicted maximum (220-age) [304]. Each cyclist's second ventilatory threshold (VT_2) was determined from the gas-exchange data collected during incremental exercise (ParvoMedics' TrueOne® 2400, Utah, USA), using the ventilatory equivalents methods [267].

The cyclists refrained from exercise for 48 h prior to the maximal exercise test and experimental trial, and avoided consumption of alcohol or caffeine in the previous 24 h, and during the exercise and post-exercise sample period. Pre-exercise blood samples were collected from the cyclists after an overnight fast. The cyclists then consumed a standardised breakfast (2000 kJ) of cereal, milk, and juice and commenced the exercise

trial at 0800 hrs. The cyclists performed exercise for 2 h at 90 % VT_2 (Monark Ergomedic 828 E, Sweden). Cardiac rate and rhythm were monitored continuously throughout exercise, and cardiac rate was recorded each minute. Pulmonary gas exchange was sampled for 4 min at 20-min intervals to ensure that the cyclists were exercising at the work rate associated with 90 % VT_2 and that no drift in oxygen uptake occurred. The cyclists were allowed to drink water *ad libitum* during exercise. The cyclists were provided with a standardised meal (4500 kJ) of sandwiches, and a milkshake at 2.5 h post-exercise.

Blood collection

Venous blood samples were collected pre-exercise, immediately post-exercise, 2 h, 4 h, 6 h, 8 h, and 24 h post-exercise. Blood was collected into tubes containing K_3EDTA (Becton Dickinson, New Jersey, USA). One tube was immediately centrifuged at 1000 × *g* for 15 min. Plasma was stored in aliquots at -80°C. The remaining blood sample was used for the cytokine stimulation assay.

Plasma cortisol analysis

Plasma cortisol was analysed by ultra-high-performance liquid chromatography tandem mass spectrometry described elsewhere [290]. Briefly, plasma samples were treated with 1 M HCl at room temperature to displace cortisol and cortisone from binding proteins. To each sample, the multi-analyte internal standard (d4-dexamethasone, d2-11-deoxycortisol and d2-cortisone) and H_2O were added before loading onto activated Oasis HLB 1 cc (30 mg) solid phase extraction cartridges (Waters, Milford, USA). Samples were eluted with 100 % ethyl acetate, dried at 50°C and reconstituted in 45 % methanol with 2 mmol/L ammonium acetate and 0.1 % formic acid. The flow rate of the UHPLC analysis was 0.4 mL/min, sample injection volume 20 μL, and column temperature 50°C. The tandem mass spectrometer was operated in positive mode electron spray ionisation. The total analytical run time on the UHPLC MS/MS was 3 min.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Milan, Italy) and then counted (Countess® Automated Cell Counter, Life Technologies, Carlsbad, CA). Cells were cultured at a concentration of 1×10^6 cells/mL and stimulated with 1 μg/mL of the mitogen phytohaemagglutinin (PHA) for 72 h. Following incubation, the supernatant was removed and stored at −80°C for subsequent analysis. Th1, Th2 and Th17 cytokine concentrations were quantified using a commercially available cytometric bead array kit (BD Pharmingen, San Diego, CA) to measure the concentrations of IL-2, IL-4, IL-6, IL-10, TNF, INF-γ and IL-17A from supernatant of stimulated samples. In the absence of stimulation with PHA, the concentrations of these cytokines was below the detection limit of the assay.

Statistics

Prior to statistical analysis, all data were tested to determine if they were normally distributed. Data that were normally distributed (Figure 3.1) were analysed using one-way ANOVA with repeated measures to determine any change over time, with a Bonferroni correction applied post hoc when significance was evident. These data are presented as mean ± SEM. Data that were not normally distributed (Table 3.3) were analysed using the non-parametric Friedman's ANOVA and Wilcoxon signed-rank test. These data are presented as median ± interquartile range. The Bonferroni correction was used for all multiple comparisons, with significance set at $p < 0.008$ ($p < 0.05$ divided by the number of time points). All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, USA). Data was further analysed for 'clinical' significance using magnitude-based inferences [305]. All qualitative analyses based on effect size were conducted using a modified statistical spreadsheet (available at www.sportsci.org/resource/stats/xPostOnlyCrossover.xls) [306]. Measures were log-transformed before analysis to symmetrically distribute the non-normally distributed data. Magnitudes of effects were determined by standardisation of the log-transformed variable. Between-time point standardized differences and effect sizes for all values were calculated using the pooled standard deviation [307]. Threshold values for Cohen Effect Size statistics were > 0.2 (small), > 0.5 (moderate), and > 0.8 (large). For between-time point

comparisons, probabilities were calculated to establish whether the true (unknown) differences were lower, similar or higher than the smallest worthwhile difference or change. The smallest worthwhile difference for each variable was derived by standardisation: 0.20 multiplied by the pooled between-subject standard deviations at each time point compared to pre-exercise, based on Cohen's Effect Size principle [307]. Quantitative chances of higher or lower differences were evaluated qualitatively as follows: < 1 %, almost certainly not; 1–5 %, very unlikely; 5–25 %, unlikely; 25–75 %, possible; 75–95 %, likely; 95–99 %, very likely; > 99 %, almost certain. If the chance of higher or lower differences was > 5 %, the true difference was assessed as unclear. Otherwise, the change was interpreted as the observed chance [305].

Results

Physical characteristics and exercise values

Physical characteristics of the cyclists are presented in Table 3.1. Mean heart rate for the 2 h exercise bout was 161 ± 3 beats.min^{-1}, and mean exercise intensity associated with 90 % VT_2 occurred at 75.1 ± 0.9 % of $\dot{V}O_2$peak.

Table 3.1. Physical characteristics and aerobic capacity of the cyclists

Subject Characteristics (n=10)	**Mean ± SEM**
Age (years)	24.2 ± 1.7
Height (m)	1.82 ± 0.02
Body mass (kg)	73.8 ± 2.0
BMI (kg.m^{-2})	22.2 ± 0.4
Absolute $\dot{V}O_2$peak (L.min^{-1})	4.83 ± 0.1
Relative $\dot{V}O_2$peak (mL.kg^{-1}.min^{-1})	65.9 ± 2.3
Training (km/week)	292.5 ± 28.4

Data are mean ± standard error of the mean. BMI: body mass index. $\dot{V}O_2$peak: peak oxygen uptake. Training: volume in kilometres performed during training per week.

Plasma cortisol

Figure 3.1 displays plasma cortisol concentration following prolonged heavy-intensity exercise. There was no statistically significant change immediately or 2 h post-exercise. Plasma cortisol concentration significantly decreased 4 h post-exercise ($p < 0.001$), when compared with the resting pre-exercise concentration, and remained significantly decreased at 6 h post-exercise ($p < 0.01$), 8 h post-exercise ($p < 0.001$) and 24 h post-exercise ($p < 0.01$).

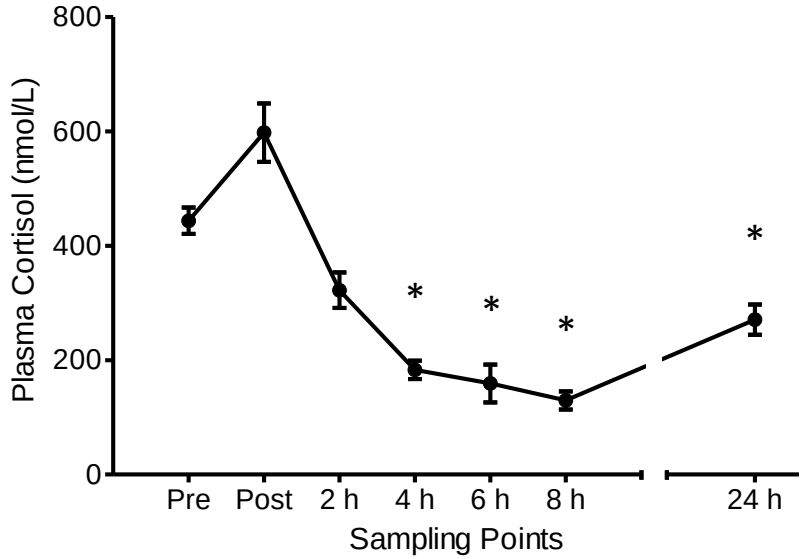

Figure 3.1. Plasma cortisol concentration before and after prolonged heavy-intensity cycling. Data represent mean ± SEM. *, significantly change compared to 'Pre-Exercise' ($p < 0.05$)

Magnitude-based inferences revealed that compared to pre-exercise, plasma cortisol concentration was likely higher immediately post-exercise. The chances that the true difference in cortisol was higher/trivial/lower were 94/5/1%. Cortisol was very likely lower at 2 h post-exercise (1/2/97 %). Furthermore, compared to pre-exercise, cortisol was almost certainly lower at 4 h (0/0/100 %), 6 h (0/0/100 %), and 8 h (0/0/100 %). At 24 h post-exercise, cortisol was likely lower (4/12/84 %) than pre-exercise values (Table 3.2).

Table 3.2. Qualitative analysis of changes in cortisol concentration following prolonged heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
% Difference	39.4	-24.6	-58.3	-63.0	-70.0	-38.8
(90% CL)	(13.2; 65.6)	(-39.8; -9.5)	(-64.7; -52.0)	(-76.7; -49.3)	(-76.8, -63.3)	(-48.0; -29.6)
Effect Size	1.3 (large)	-1.4 (large)	-4.2 (large)	-3.2 (large)	-5.1 (large)	-2.2 (large)
% Chance for value to be higher/trivial/lower than Pre	94/5/1	1/2/97	0/0/100	0/0/100	0/0/100	4/12/84
Qualitative inference	Likely higher	Very likely lower	Almost certainly lower	Almost certainly lower	Almost certainly lower	Likely lower

Data presented as percentage difference compared to the pre-exercise concentration. CL: Confidence level.

Table 3.3. T helper cytokine release before and after a bout of prolonged heavy-intensity exercise

	Pre-exercise	Post-exercise	2 h	4 h	6 h	8 h	24 h	P value
IL-2	18.9 (3.9, 59.8)	11.5 (2.8, 24.0)	11.2 (2.5, 51.7)	65.8 (8.0, 110.0)	50.7 (10.6, 107.0)	47.4 (13.6, 96.3)	7.1 (1.7, 162.0)	0.141
TNF	2.0 (1.5, 2.4)	1.8 (1.4, 2.0)	1.8 (1.5, 2.1)	2.3 (2.0, 3.3)	2.9 (1.7, 3.9)	2.0 (1.6, 2.6)	1.9 (1.4, 2.8)	0.488
IFN-γ	1.1 (1.1, 1.3)	1.1 (1.0, 1.2)	1.2 (1.1, 1.4)	1.3 (1.1, 2.4)	1.6 (1.2, 2.3)	1.8 (1.1, 4.8)	1.1 (1.0, 1.4)	0.103
IL-4	2.0 (1.9, 2.1)	2.1 (2.0, 2.2)	2.1 (1.9, 2.3)	2.0 (1.9, 2.2)	2.0 (1.9, 2.1)	2.0 (2.0, 2.2)	1.9 (1.9, 2.0)	0.227
IL-6	347.7 (14.3, 835.8)	142.7 (32, 1061.8)	98.3 (30.5, 433.4)	249.9 (112, 706.2)	458.4 (125, 1006)	718.2 (95.7, 990.3)	131.7 (49.9, 606.0)	0.039
IL-10	4.4 (1.8, 7.5)	2.7 (1.8, 7.2)	4.6 (1.8, 7.4)	5.2 (2.2, 16.1)	11.5 (2.7, 17.0)	6.6 (2.2, 19.3)	3.7 (1.5, 30.0)	0.126
IL-17A	2.8 (2.8, 3.0)	2.9 (2.5, 3.3)	2.7 (2.7, 3.2)	2.8 (2.8, 3.2)	2.8 (2.6, 3.1)	3.0 (2.7, 3.1)	2.8 (2.6, 3.0)	0.500

Th1 cytokines: IL-2, TNF, IFN-γ. Th2 cytokines: IL-4, IL-6, IL-10. Th17 cytokine: IL-17A. Data represent median (1st and 3rd quartile).

T helper cell cytokines

There were no statistically significant changes in PHA-stimulated production of the T helper 1 cytokines IL-2, TNF, and IFN-γ, T helper 2 cytokines IL-4 and IL-10, and T helper 17 cytokine IL-17A following exercise (Table 3.3). A significant time effect was observed for IL-6; however, post hoc analysis revealed no significant change at individual time points after exercise compared with the pre-exercise IL-6 concentration.

Table 3.4 displays changes in Th1 cytokine concentrations. Compared with pre-exercise values, the concentration of IL-2 was likely higher than pre-exercise at 4 h (85/15/0 %) and 8 h (90/10/0 %) post-exercise. The concentration of TNF was also likely higher than pre-exercise values at 4 h post-exercise (82/17/1 %). Changes in IFN-γ concentration were unclear at every time point post-exercise.

Compared to pre-exercise, the concentration of IL-4 was likely higher immediately post-exercise (79/18/3 %), while the difference in IL-4 during the remaining recovery period were unclear (Table 3.5). Compared to pre-exercise, the IL-6 concentration was possibly higher at 4 h (68/29/3 %), possibly lower at 6 h (4/42/54 %), and likely higher at 8 h post-exercise (94/6/0 %). Compared to pre-exercise, IL-10 concentration was likely higher at 4 h post-exercise (77/23/0 %).

IL-17A concentration compared to pre-exercise was possibly higher than pre-exercise at 8 h post-exercise (73/25/2 %) (Table 3.6).

Table 3.4. Qualitative analysis of changes in Th1 cytokine concentrations following prolonged heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
IL-2						
% Difference	69.3	-4.0	162.2	142.4	115.3	132.6
(90% CL)	(-75.7; 214.4)	(-33.0; 25.1)	(42.5; 281.9)	(64.0; 220.7)	(34.0; 196.5)	(-41.5; 306.6)
Effect Size (rating)	-0.1 (trivial)	0.04 (trivial)	0.5 (moderate)	0.5 (moderate)	0.3 (small)	0.5 (moderate)
% Chance for value to be higher/trivial/lower than Pre	7/49/44	1/77/22	85/15/0	26/59/15	90/10/1	4/94/2
Qualitative inference	Unclear	Trivial	Likely higher	Unclear	Likely higher	Trivial
TNF						
% Difference	-3.2	80.7	45.4	145.2	11.4	20.5
(90% CL)	(-24.7; 18.2)	(-55.9; 217.4)	(-1.1; 92.0)	(-43.8; 334.3)	(-9.4; 32.2)	(-17.4; 58.4)
Effect Size (rating)	-0.4 (small)	0.5 (moderate)	0.6 (moderate)	0.7 (moderate)	0.1 (trivial)	0.2 (small)
% Chance for value to be higher/trivial/lower than Pre	6/47/47	56/31/14	82/17/1	69/21/11	44/46/10	60/26/14
Qualitative inference	Unclear	Unclear	Likely higher	Unclear	Unclear	Unclear
IFN-γ						
% Difference	5.2	7.3	11.8	25.5	77.0	21.8
(90% CL)	(-32.7; 43.2)	(-28.2; 42.8)	(-11.9; 35.5)	(-4.6; 55.6)	(-0.0; 154.1)	(-35.5; 79.2)
Effect Size (rating)	-0.5 (moderate)	-0.4 (small)	-0.2 (small)	-0.1 (trivial)	0.2 (small)	-0.03 (trivial)
% Chance for value to be higher/trivial/lower than Pre	11/28/61	11/47/42	18/77/6	45/50/5	50/43/7	33/54/14
Qualitative inference	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear

Data presented as percentage difference compared to the pre-exercise concentration. CL: Confidence level.

Table 3.5. Qualitative analysis of changes in Th2 cytokine concentrations following prolonged heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
IL-4						
% Difference	5.4	5.1	4.2	-0.1	2.6	-3.4
(90% CL)	(-0.4; 11.2)	(-3.2; 13.4)	(-3.6; 11.9)	(-4.3; 4.0)	(-3.9; 9.2)	(-8.9; 2.0)
Effect Size (rating)	0.5 (moderate)	0.4 (small)	0.3 (small)	-0.1 (trivial)	0.2 (small)	-0.6 (moderate)
% Chance for value to be higher/trivial/lower than Pre	79/18/3	66/23/10	62/28/11	33/26/41	21/43/35	14/23/62
Qualitative inference	Likely higher	Unclear	Unclear	Unclear	Unclear	Unclear
IL-6						
% Difference	250.8	159.2	640.3	397.5	461.1	359.7
(90% CL)	(79.8; 581.3)	(-123.0; 441.4)	(-195.6; 1476.1)	(109.1; 686.0)	(87.8; 834.5)	(-75.3; 794.7)
Effect Size (rating)	0.1 (trivial)	-0.6 (moderate)	-0.2 (small)	0.2 (small)	0.4 (small)	0.04 (trivial)
% Chance for value to be higher/trivial/lower than Pre	39/54/7	11/60/29	68/29/3	4/42/54	94/6/0	35/64/1
Qualitative inference	Unclear	Unclear	Possibly higher	Possibly lower	Likely higher	Trivial
IL-10						
% Difference	10.9	46.7	63.7	116.5	139.7	153.0
(90% CL)	(-27.8; 49.7)	(-28.5; 121.8)	(19.0; 108.4)	(3.9; 229.1)	(49.5; 229.8)	(10.2; 295.8)
Effect Size (rating)	-0.1 (trivial)	0.4 (small)	0.5 (moderate)	0.6 (moderate)	0.8 (large)	0.9 (large)
% Chance for value to be higher/trivial/lower than Pre	7/69/25	26/70/4	77/23/0	46/43/11	46/53/0	25/72/3
Qualitative inference	Unclear	Trivial	Likely higher	Unclear	Trivial	Trivial

Data presented as percentage difference compared to the pre-exercise concentration. CL: Confidence level.

Table 3.6. Qualitative analysis of changes in Th17 cytokine IL-17A concentration following prolonged heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
IL-17A						
% Difference	1.9	-1.2	5.2	4.8	8.4	-2.0
(90% CL)	(-3.8; 7.7)	(-10.3; 7.9)	(-6.4; 16.9)	(-6.5; 16.0)	(-5.3; 22.0)	(-11.4; 7.4)
Effect Size (rating)	0.1 (trivial)	-0.1 (trivial)	0.3 (small)	0.2 (small)	0.4 (small)	-0.4 (small)
% Chance for value to be higher/trivial/lower than Pre	29/62/9	15/42/44	51/35/15	17/30/53	73/25/2	31/32/37
Qualitative inference	Unclear	Unclear	Unclear	Unclear	Possibly higher	Unclear

Data presented as percentage difference compared to the pre-exercise concentration. CL: Confidence level.

Discussion

This is the first study to analyse changes in Th1, Th2 and Th17 cytokine production during recovery from exercise. It is established that exercise intensity and duration influence the cortisol response to exercise. Previously, cycle exercise of comparable intensity (~75 % $\dot{V}O_2$peak) and duration (2 h) resulted in a significant increase in cortisol concentration immediately post-exercise [308]. Although traditional data analysis in the present study did not reveal any statistically significant increase in plasma cortisol immediately post-exercise, quantitative methods used suggested an increase in plasma cortisol concentration was likely, and given results of previous research of similar exercise duration and intensity, this result would be expected.

Plasma cortisol concentration decreased below pre-exercise levels from 4 h to 24 h post-exercise. While the initial decrease (which occurred early in the afternoon) may be explained by diurnal variation [309], plasma cortisol concentration remained below resting values at 24 h post-exercise. This low plasma cortisol concentration may be clinically important when considering the repetitive nature of endurance training day after day. This result may indicate that the cyclists were well rested following the exercise bout, and would have been able to respond positively to an additional bout of exercise on the second morning.

Using traditional data analysis, there were no statistically significant changes in T helper cell cytokine secretion at any time point during the post-exercise period in the present study (Table 3.3). However, analysis using magnitude-based inferences suggested a multitude of changes following exercise. Compared to pre-exercise, IL-2 (Th1) production was likely higher at 4 h and again at 8 h post-exercise (Table 3.4), while TNF (Th1) production was likely higher at 4 h post-exercise (Table 3.4). IL-4 (Th2) production was likely higher immediately post-exercise (Table 3.5), while IL-6 (Th2) production was possibly higher at 4 h, possibly lower at 6 h, and likely higher at 8 h post-exercise (Table 3.5). IL-10 (Th2) production was likely higher at 4 h post-exercise (Table 3.5), while IL-17A (Th17) production was possibly higher at 8 h post-exercise (Table 3.6).

Previously, intracellular cytokines IL-2 (Th1), IFN-γ (Th1), and IL-4 (Th2) produced by $CD4^+$ T cells were used to define T helper cell types and their functional changes. Using this method, a Th1-to-Th2 shift was described following exercise and hormone infusion [155, 167, 168, 310]. This 'shift' occurs when IL-2- and/or IFN-γ-producing T helper cells (Th1) decrease in cell concentration, while IL-4-producing T helper cells (Th2) remain unchanged. In the present study, no change in IL-2 and IFN-γ occurred immediately post-exercise (Table 3.4), while IL-4 concentration increased at the same time point (Table 3.5). Hence, a shift towards Th2 was observed immediately post-exercise.

Although single intracellular cytokine analysis of T helper cells provides a concise explanation of the Th response to exercise, it neglects to consider the magnitude of cytokines involved in the T helper cell network. Cytokines are not solely secreted from T helper cells, but include cytotoxic T cells, natural killer cells, dendritic cells, macrophages and even skeletal muscle [311, 312]. In the present study, stimulated cytokine secretion both increased and decreased occurred immediately following exercise (IL-4), and up to 8 hours post-exercise (IL-2, IL-6 and IL-17A). This presents an intricate pattern of changes to consider. In particular, at 4 h post-exercise, the Th1 cytokines IL-2 and TNF, and the Th2 cytokines IL-6 and IL-10 were all increased. This concomitant increase and balancing of Th1 and Th2 cytokines has been observed previously by measuring intracellular cytokine production from unstimulated peripheral blood mononuclear cells following 30 min of moderate-intensity cycling [312]. In contrast, the present study examined the functional ability of peripheral blood mononuclear cells to respond to a stimulus (PHA). Interestingly, the release of multiple cytokines was elevated at 4 h post-exercise. These responses demonstrate that exercise-induced stress can prime peripheral blood mononuclear cells to respond to stimulation [313].

Recently, analysis by magnitude-based inference has received greater use in exercise science and clinical settings. As previously stated, using traditional data analysis, there were no statistically significant changes in stimulated T helper cell cytokine secretion at any time point during the post-exercise period in the present study (Table 3.3). However, analysis using magnitude-based inferences suggested various changes following exercise,

which may prove clinically significant. This approach may provide insights into what are clinically important, yet statistically non-significant results in physiological and immunological processes. This study assists in highlighting the importance of considering non-traditional methods for data analysis when dealing with small sample sizes, with high between-subject variability.

Finally, regarding changes in individual cell populations within the isolated PBMC population; previous research has demonstrated changes in circulating concentrations of PBMC subsets in response to exercise [1]. The proportion of different cell types making up the isolated PBMC population may change at different time points following exercise, potentially impacting on the results. Changes in the proportion of discrete cell subtypes with exercise could have altered the number of cells capable of responding to the mitogen and secreting cytokines. PBMCs consist of monocytes (10 – 30%), dendritic cells (1 – 2%), and primarily of lymphocytes (70 – 90%). T cells (45 – 70% of PBMCs), B cells (up to 15% of PBMCs), and NK cells (up to 15% of PBMCs) make up the predominant portions of the lymphocyte population [47]. Whereas, those cells excluded during PBMC isolation consist of polymorphonuclear cells (PMNs) such as neutrophils and eosinophils [49]. Of the PBMC populations, NK cells are the most responsive to the stress response, followed by T cells, monocytes, dendritic cells, and then B cells [104, 111]. As such, with a set number of cells collected at each time point (1×10^6 cells/mL), it is likely that following exercise the proportion of NK and T cells increased, while the proportion of monocytes, dendritic cells and B cells decreased, reaching a peak difference between immediately, and 2 h post-exercise. This may have an effect on cytokine release, as different cells produce different cytokines [314]. It could be argued that changes in cytokine production are less due to the direct effect of catecholamines and cortisol on cellular function, rather the change in representation in circulating cell types driving the change in perceived function/cytokine release.

The changes that occurred in T helper cell cytokine release suggest a multifactorial, prolonged response to heavy endurance exercise. The fluctuations in cytokine production that occurred during recovery from exercise could have important implications for

resistance to illness in athletes. While intracellular cytokine analysis following stimulation provides a concise explanation of the Th1/Th2 shift, the current study suggests a much more complex response to exercise.

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Author Disclosure Statement

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4. STUDY II – DENDRITIC CELL AND γδ T CELL RESPONSES TO PROLONGED HEAVY ENDURANCE EXERCISE

Abstract

The purpose of this study was to assess alterations in circulating dendritic cell sub-populations, γδ T cell sub-populations and memory phenotypes after prolonged heavy-intensity exercise. Ten well-trained endurance cyclists (mean ± SD: age 24.0 ± 4.1 years; height 1.81 ± 0.05 m; body mass 73.3 ± 5.8 kg; peak oxygen uptake 60.7 ± 4.6 mL.kg^{-1}.min^{-1}) performed 2 h of cycling exercise at 90% of their second ventilatory threshold. Blood samples were collected before exercise, immediately post-exercise, 1 h, 2 h, 4 h, and 6 h post-exercise. Flow cytometry was used to examine γδ T cell subsets, dendritic cell phenotypes, memory phenotypes and receptor expression. A significant decrease in cell concentration was evident in γδ T cells and the δ2 subset up to 4 h post-exercise. Phenotypical differentiation revealed a significant decrease in naïve and central memory δ2 cells. Additional analysis revealed preferential egress of γδ T cells expressing CD11a^{high} and CD62L^{high}. Finally, a significant decrease was evident in total dendritic cells at 1 h post-exercise, together with decreases in myeloid dendritic cells and CD14$^{-/low}$CD16^{+} dendritic cells up to 2 h post-exercise. These results suggest an exercise-stress-induced egress of γδ T cells from the circulation with greater propensity for tissue and lymph node homing for a period of 4 h after intense exercise.

Introduction

Stress-induced leucocyte redistribution is a well-documented phenomenon [102]. The rapid ingress of neutrophils and lymphocytes into circulation in response to stressors such as psychological stress [104], exercise [1], and surgery [105] follows a distinct pattern. This involves a redistribution of cells from areas of storage into the blood vessels (ingress) and out to the tissues (egress) [106, 107]. Specifically, prolonged heavy-intensity exercise—defined here as sustainable exercise of duration > 1.5 h, above the anaerobic threshold—induces cell redistribution of lymphocyte and granulocytes from immediately, until up to 8 h, following exercise [1, 107]. Changes in cellular redistribution and function of neutrophils, T cells, B cells, and natural killer cells have been thoroughly characterized during the immediate response to, and recovery from, prolonged heavy-intensity exercise [1, 197, 308]. This research has led to the 'open window' hypothesis of risk of illness with exercise [107]. To date, changes in γδ T cells and dendritic cells in response to exercise have received less attention, and may provide further information as to the cellular response to exercise.

γδ T cells have been labelled 'the bridge between innate and adaptive immunity' [78]. These cells perform MHC-independent activation, T cell receptor-dependent recognition of conserved antigens, and can act as professional antigen presenting cells. They also have protective memory and reactive memory capabilities [78, 79]. Two major subsets (δ1 and δ2)—which are further divided into four memory phenotypes (according to presence of CD45RA and CD27)—are used to distinguish the specific roles of γδ T cells [79]. Alterations in naïve ($CD45RA^{+}/CD27^{+}$), central memory ($CD45RA^{-}/CD27^{+}$), effector memory ($CD45RA^{-}/CD27^{-}$) and terminally differentiated effector memory ($CD45RA^{+}/CD27^{-}$) phenotypes can be characterized. Previously, acute exercise bouts of 20 to 30 minutes caused egress of γδ T cells immediately post-exercise [104, 160]. More detailed phenotypical analysis revealed psychological stress mobilises cells with high cytotoxic capabilities, tissue homing potential, and the capacity for rapid, innate-like target recognition [163]. These previous studies examined short duration stress and exercise, with the immediate ingress of cell subsets into circulation. The present study aimed to analyse changes in these cell types following a much more prolonged stress stimulus, and monitor the redistribution (ingress and egress) during the 6-hour period following exercise.

Dendritic cells (DCs) functioning as professional APCs in the blood appear to have immature/precursor characteristics, because they undergo a maturation process after entering the tissue [63]. As such, exercise-induced redistribution may have considerable effects on dendritic cell function. Myeloid DCs ($HLA.DR^+/lin^-/CD11c^+$) are able to stimulate allogen- and antigen-specific T cell responses. By contrast, plasmacytoid DCs ($HLA.DR^+/lin^-/CD123^+$) are closely involved in regulatory T cell and cytotoxic T cell activation, and regularly migrate to secondary lymphoid organs. $CD16^+$ DCs ($HLA.DR^+/lin^-/CD11c^+/CD16^+$) are derived from monocytes, and activate T helper cells in an allogeneic fashion [65, 74]. Together, these cells play a crucial role in initiating and regulating both cellular and humoral immune responses. Previous research has revealed that following a marathon, myeloid DCs increase significantly, while plasmacytoid DCs decreased significantly below pre-exercise values [164]. Following a 1-hour ice-hockey training session, both myeloid and plasmacytoid DCs increased significantly immediately after exercise [165]. Neither of these studies examined $CD16^+$ DCs, and included only pre-, post-, and 24 h post-exercise samples to examine redistribution. Further research is warranted to identify and understand changes in redistribution of this rarely examined cell type during the early phase of recovery from exercise.

Very little research is currently in the literature examining exercise- or stress-induced changes in γδ T cells and dendritic cells. Importantly, these cells are involved heavily in regulation, antigen presentation and innate recognition. Prolonged heavy-intensity exercise, in the form of single acute bouts or extended periods of heavy training, has been suggested to induce short term immune suppression [1, 296]. As such, the aim of the study was to analyse γδ T cell and dendritic cell redistribution following an acute stressor of prolonged heavy-intensity exercise.

Methods

Subjects

Ten well-trained male cyclists volunteered to participate in the present study. Physical characteristics of the cyclists are presented in Table 4.1. Subjects were excluded if they exhibited any cardiovascular, metabolic, neurological, immunological or autoimmune disorders. The cyclists provided written informed consent for participation in the study, which was approved by the Bond University Human Research Ethics Committee.

Table 4.1. Physical characteristics, aerobic capacity, threshold performance data, and physiological variables during exercise of the cyclists

Subject Characteristics (n=10)	Mean ± SD
Age (years)	24.0 ± 4.1
Height (m)	1.81 ± 0.05
Body mass (kg)	73.3 ± 5.8
BMI ($kg.m^{-2}$)	22.3 ± 1.4
Absolute $\dot{V}O_2$peak ($L.min^{-1}$)	4.4 ± 0.4
Relative $\dot{V}O_2$peak ($mL.kg^{-1}.min^{-1}$)	60.7 ± 4.6
Peak Power (W)	445 ± 41
Peak Power-to-weight (W/kg)	6.1 ± 0.5
VT_1 Power (W)	287 ± 28
VT_1 Power-to-weight (W/kg)	3.9 ± 0.3
VT_2 Power (W)	346 ± 27
VT_2 Power-to-weight (W/kg)	4.7 ± 0.4
Training (km/week)	348 ± 57
Mean HR ($beats.min^{-1}$)	153 ± 11
Mean $\dot{V}O_2$ ($L.min^{-1}$)	3.4 ± 0.3
Mean Cadence ($revolutions.min^{-1}$)	94 ± 4
Mean Power output (W)	296 ± 18

Data are mean ± standard deviation. BMI: body mass index. $\dot{V}O_2$peak: peak oxygen uptake. VT_1: first ventilator threshold. VT_2: second ventilator threshold. Training: volume in kilometres performed during training per week. HR: heart rate.

Experimental Design

The cyclists visited the laboratory on three occasions; for a screening/familiarization session, a maximal exercise test, and a constant load test with blood sampling before and after exercise. Each session was separated by at least three days during which time they were permitted to train as normal. Throughout the testing period, cyclists were required to report any upper respiratory or gastrointestinal symptoms. Peak oxygen consumption ($\dot{V}O_2$peak) was determined during a graded exercise test in which the cyclists were allowed to choose their preferred cadence within the range of 70–90 rev.min^{-1}. The test was performed on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The cyclists commenced cycling at 90 W for 4 min; thereafter intensity increased by 30 W/min until volitional fatigue and/or when the cyclists could not maintain their cadence above 70 rev.min^{-1}. The criteria to determine maximal effort at the end of a test were: a plateau in $\dot{V}O_2$ (defined as an increase in $\dot{V}O_2$ values of less than 1.5 ml.kg^{-1}.min^{-1} between two consecutive 1-min periods in the final part of the test), a maximal value of respiratory exchange ratio ≥ 1.1, a maximal heart rate value above 95% of the age-predicted maximum (220-age) [304]. Each cyclist's first (VT_1) and second (VT_2) ventilatory threshold was determined from the gas-exchange data collected during incremental exercise (Medgraphics® 2400, Utah, USA), using the ventilatory equivalents methods [267].

The cyclists refrained from exercise for 48 h prior to the maximal exercise test and the experimental trial, and avoided consumption of alcohol or caffeine in the previous 24 h, and during the exercise and post-exercise sample period. Pre-exercise blood samples were collected from the cyclists after an overnight fast. The cyclists then consumed a standardized breakfast (2000 kJ) of cereal, milk, and juice and commenced the experimental trial at 8 a.m. hrs. The cyclists performed exercise for 2 h at 90% VT_2 on a stationary cycle ergometer (Monark Ergomedic 828 E, Sweden). Heart rate was monitored continuously throughout the exercise. Pulmonary gas exchange was sampled for 4 min at 20-min intervals to ensure that the cyclists were exercising at the work rate associated with 90% VT_2 and that no drift in oxygen consumption occurred. The cyclists were allowed to drink water *ad libitum* during exercise. Immediately after exercise, a venous blood sample

was collected. Additional blood samples were collected 1 h, 2 h, 4 h, and 6 h post-exercise. The cyclists were provided with a standardized meal following the blood collection at 2 h post-exercise. The meal consisted of sandwiches and a milkshake (4500 kJ).

Flow cytometry

Venous blood was sampled from an antecubital vein and collected into blood collection tubes containing K_3EDTA (BD Vacutainer, New Jersey, USA). Samples were analysed to determine a full blood cell count (Coulter HmX Hematology Analyzer, Beckman Coulter). Cells were identified by immunofluorescent antibody staining of whole blood using four-color flow cytometry (FACS-Calibur, BD Biosciences). Flow cytometric analysis was completed within six hours of blood collection.

Lymphocyte subsets

Total γδ T cells, δ1 and δ2 subsets, memory phenotypes, and surface molecules were determined using the following antibodies; CD3 PerCP (BD Biosciences), Vδ-1 TCR FITC (Thermo Scientific), γδ TCR APC, Vδ-2 TCR APC, CD27 FITC, CD27 PE, CD45RA APC, CD11a FITC, CD62L PE, CD94 FITC (BD Pharmingen) [163]. Briefly, 100 µL whole blood were incubated with appropriate amounts of antibody (as determined by titration) for 30 min in the dark at room temperature. Erythrocytes were subsequently lysed using FACS lysing solution (BD Bioscience). Following lysis, samples were centrifuged (350 × *g*, 5 min, at room temperature). Samples were then washed and fixed in 1% paraformaldehyde. Fixed preparations were analysed immediately, collecting a minimum of 100,000 total events from each sample. Appropriate, matched isotype controls were used to set negative staining criteria. The FACS-Calibur flow cytometer (BD Biosciences) was calibrated weekly using Calibrite beads (BD Biosciences). Total lymphocytes were gated using forward versus side scatter. γδ T cells were then gated as the $CD3^+$ γδ TCR^+ population, δ1 T cell were gated as $CD3^+V\delta1^+$ and δ2 T cells were gated as $CD3^+$ $V\delta2^+$. Lymphocyte subset numbers were quantified using the percentage values obtained from each lymphocyte subset gate. The total lymphocyte concentration was obtained from the

full blood count. Percentage values of subsets were multiplied by the total lymphocyte concentration to determine absolute concentrations of target lymphocyte subsets.

Dendritic cell phenotypes

Total dendritic cells (tDCs), dendritic cell phenotypes (pDCs, mDCs, $CD14^{-/low}CD16^{+}$ DCs), and total monocytes were identified by the combination of HLA-DR PerCP, lineage cocktail 2 FITC (CD3, CD14, CD19, CD20, CD56), CD123 PE (BD Bioscience), CD33 APC, CD11c PE and CD16 APC (BD Pharmingen) antibodies. Expression of CD33 and HLA-DR and absence of the lineage cocktail 2 with intermediate forward (FSC) and side scatter (SSC) between those of lymphocytes and monocytes was used to determine tDCs. Dendritic cells were further defined into three subpopulations: (1) a subset of $CD123^{dim}/CD33^{high}/CD16^{-}$ cells, with strong expression of HLA-DR–mDCs; (2) a subset of $CD123^{inter}/CD33^{inter}/CD16^{+}$ cells–$CD14^{-/low}CD16^{+}$ DCs and (3) $CD123^{high}/CD33^{-/dim}/CD16^{-}$ cells–pDCs, both with intermediate expression of HLA-DR. Monocytes were identified as expressing CD33, CD14 and HLA-DR and by their forward and side scatter characteristics [289]. Absolute counts were quantified using percentage values obtained from each lymphocyte and monocyte subset gate. The total monocyte concentration was obtained from the full blood count.

Statistics

All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, USA). Data at each time point were compared using one-way ANOVA with repeated measures. Bonferroni correction was applied *post hoc* when significance was evident. Normally distributed data are presented as the mean and standard deviation (SD). The criterion for significance was set at $p<0.05$. Non-normally distributed data (as defined by testing the assumptions of normality, homogeneity of variance and sphericity) were analysed using Friedman's ANOVA. In the result of significant main effect ($p<0.05$), Wilcoxon signed-rank test was employed *post hoc* for comparisons between pre-exercise and subsequent time points. The criterion for significance was adjusted for multiple time points to $p<0.01$. Non-normally distributed data are presented as the median ± 1st and 3rd

quartile. To examine the effect of resting concentration on cellular egress; data for lymphocyte and dendritic cell subsets were also divided into “low” and “high” concentration groups according to their resting concentration. Percentage changes from pre-exercise to nadir values were included. Additionally, the relationship between pre-exercise cell concentration and nadir cell concentration was explored using Spearman rank data correlation analysis to test for correlations. $P<0.05$ was accepted as significant.

Results

Physiological variables during exercise

Mean heart rate for the 2 h exercise bout was 153 ± 11 beats.min^{-1}, while mean power was 296 ± 18 W with a mean $\dot{V}O_2$ of 3.4 ± 0.3 L.min^{-1} corresponding to 76.5 ± 2.5 % of $\dot{V}O_2$peak.

Full Blood Count

A significant time effect was evident for total lymphocytes; however *post hoc* analysis did not reveal a significant change between pre-exercise and any time point (Figure 4.1). Prolonged heavy exercise induced a significant increase in the concentration of circulating granulocytes (Figure 4.2). To examine cellular egress, data for total lymphocytes are also presented in "low" and "high" concentration groups according to resting concentration. The "low" and "high" concentration groups decreased by 4.14% and 21.07%, respectively from pre-exercise to nadir at 2 h post-exercise. When examining pre-exercise lymphocyte concentration versus the difference between pre-exercise and nadir values, there was a significant negative relationship ($r = -0.80$, $p<0.01$). Furthermore, changes in granulocyte and monocyte ingress were examined by dividing into "low" and "high" concentration groups according to their resting concentrations. For granulocytes, "low" and "high" concentration groups increased by 198.6% and 169.3%, respectively from pre-exercise to peak at 1 h post-exercise. When examining pre-exercise granulocyte concentration versus the difference between pre-exercise and peak values, no significance was found ($r = 0.16$, $p=0.66$). For monocytes, "low" and "high" concentration groups increased by 32.0% and 39.0%, respectively from pre-exercise to peak at 2 h post-exercise. When examining pre-exercise monocyte concentration versus the difference between pre-exercise and peak values, no significance was found ($r = 0.02$, $p=0.95$).

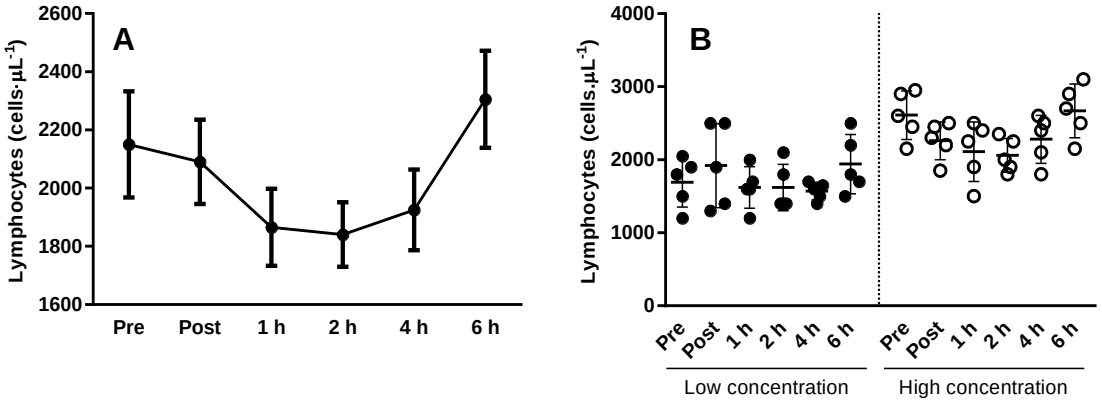

Figure 4.1. Total lymphocyte concentration (A), and lymphocyte concentration presented as individual samples divided into "low" and "high" concentration groups (B) before and after a bout of prolonged heavy-intensity exercise. Time effect: p <0.001.

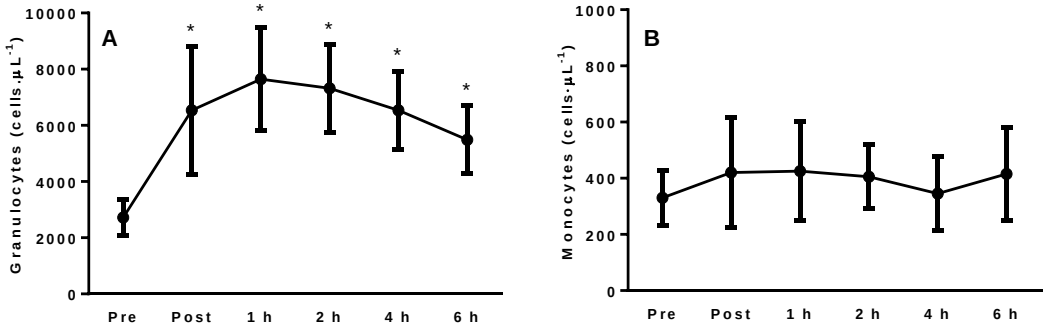

Figure 4.2. Total granulocyte (A) and monocyte (B) concentrations before and after a bout of prolonged heavy-intensity exercise. Data represent mean ± SD. Time effect: Granulocytes; p <0.001, Monocytes; p=0.178. *, significantly different from pre-exercise, p<0.05.

Total γδ T cells

Prolonged heavy exercise reduced the concentration of circulating γδ T cells (Figure 4.3). A significant time effect was evident for both γδ T cells (Figure 4.3) and the δ2 subsets (Figure 4.5). *Post hoc* analysis revealed that both γδ T cells and the δ2 subsets were significantly lower than pre-exercise values at 1 h, 2 h, and 4 h post-exercise (p<0.01). There were no significant changes in the δ1 subset at any time point. Data for total γδ T cells, and δ1 and δ2 subsets are also presented in "low" and "high" concentration groups.

The “low” and “high” concentration groups decreased by 37.9% and 47.7% (Total γδ), 6.9% and 39.1% (δ1), and 45.1% and 56.8% (δ2), respectively from pre-exercise to nadir values. When examining pre-exercise concentration versus the difference between pre-exercise and nadir values, there was a significant relationship in total γδ (r = -0.93, p<0.001), δ1 (r = -0.83, p<0.01), and δ2 (r = -0.95, p<0.001).

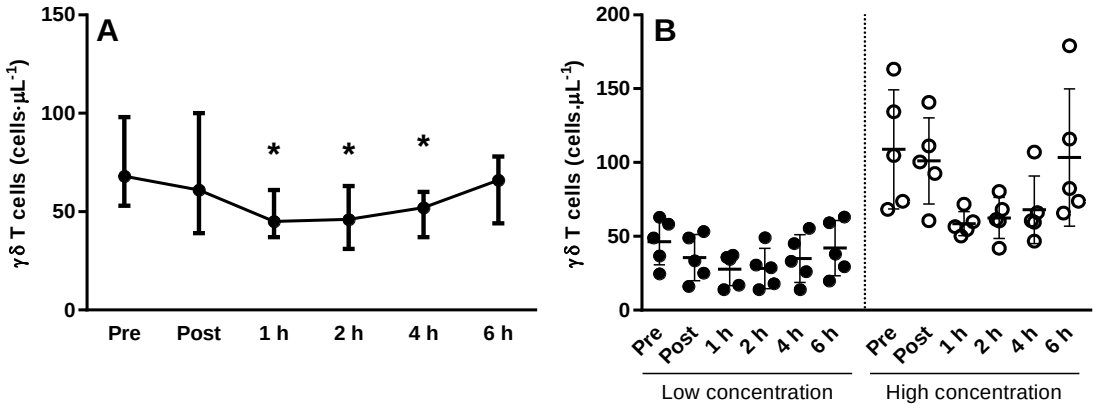

Figure 4.3. Total γδ T cell concentration (A), and individual concentrations of γδ T cells divided into “low” and “high” concentration groups (B) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). *, significantly different from pre-exercise, p<0.01.

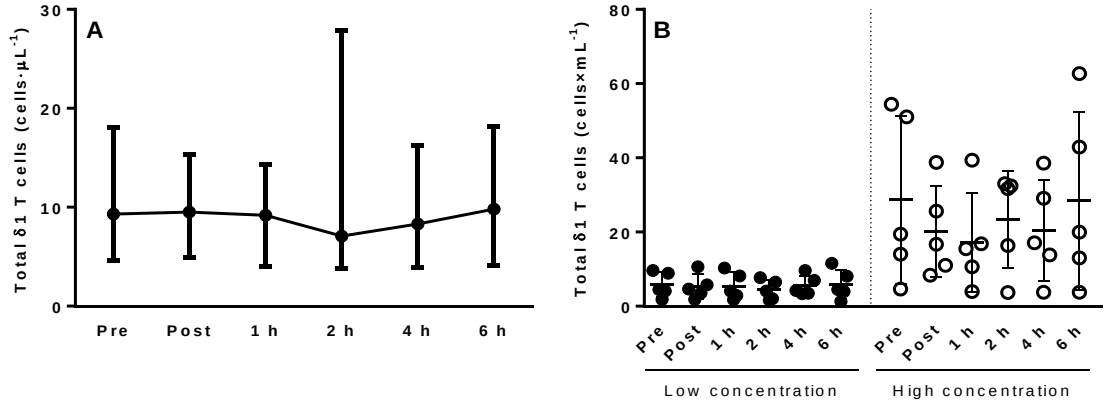

Figure 4.4. δ1 γδ T cell subset concentration (A), and individual concentrations of δ1 γδ T cells divided into “low” and “high” concentration groups (B) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile).

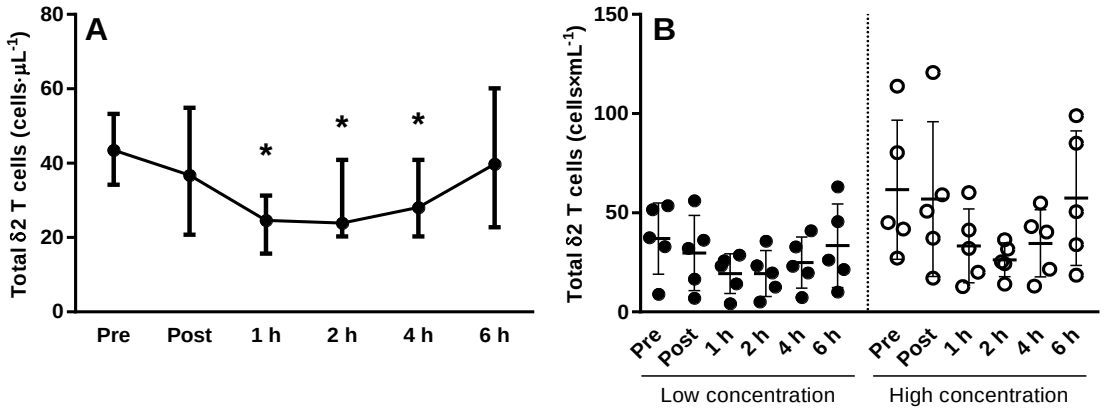

Figure 4.5. δ2 γδ T cell subset concentration (A), and individual concentrations of δ2 γδ T cells divided into "low" and "high" concentration groups (B) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). *, significantly different from pre-exercise, $p<0.01$.

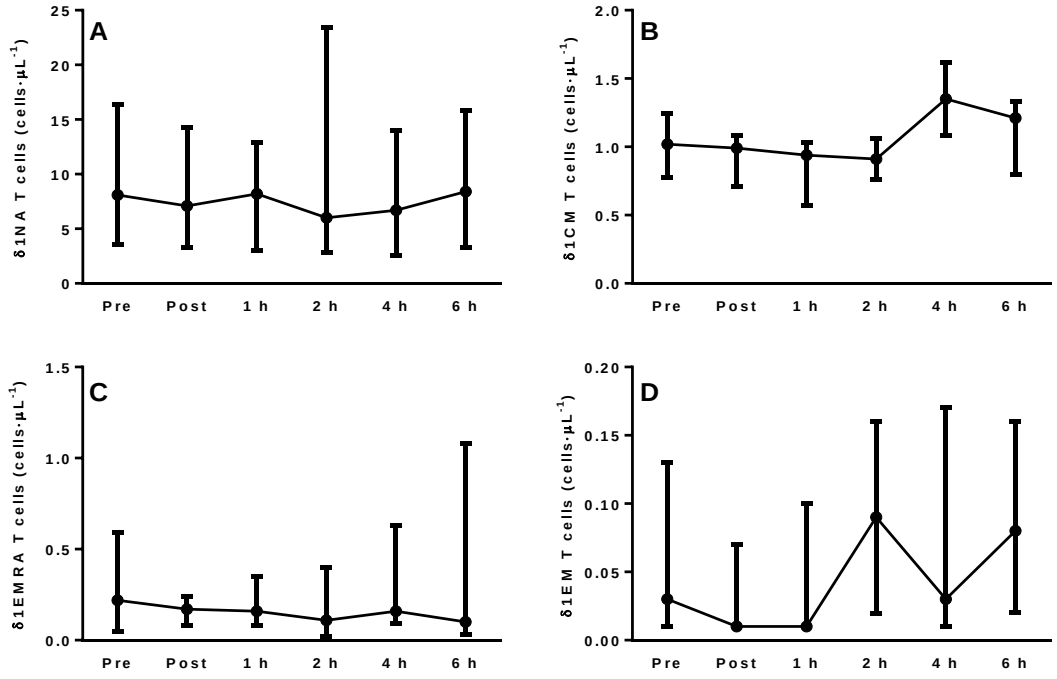

Figure 4.6. δ1 naïve (A), central memory (B), CD45RA$^+$ effector memory (C) and effector memory (D) phenotype concentrations before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). NA: naïve. CM: central memory. EM: effector memory. EMRA: CD45RA$^+$ effector memory.

δ1 and δ2 memory phenotypes

Both δ1 and δ2 γδ T cell subsets were further divided into four memory phenotypes, according to expression of CD45 and CD27 antigens. A significant time effect was evident for changes in naïve, central memory and effector memory δ2 γδ T cell memory phenotypes (Figure 4.7). *Post hoc* analysis revealed that naïve δ2 cell concentration was significantly lower than pre-exercise values at 1 h, 2 h, and 4 h post-exercise , while central memory δ2 γδ T cell concentration was significantly lower than pre-exercise values at 1 h and 2 h post-exercise ($p<0.01$).

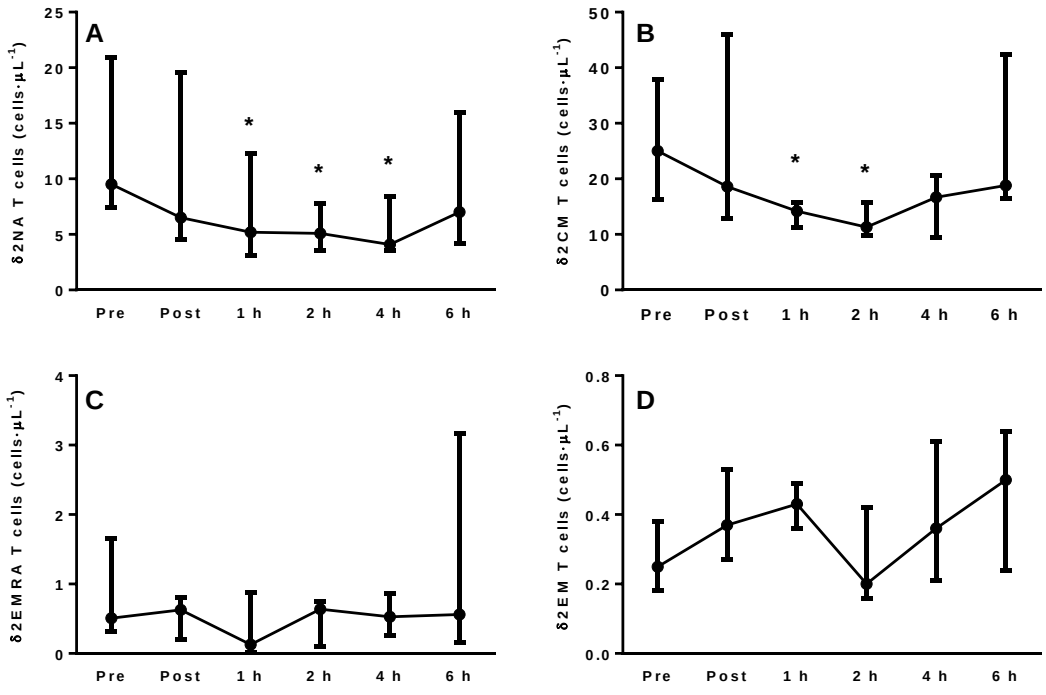

Figure 4.7. δ2 naïve (A), central memory (B), CD45RA$^+$ effector memory (C) and effector memory (D) phenotype concentrations before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). NA: naïve. CM: central memory. EM: effector memory. EMRA: CD45RA$^+$ effector memory. *, significantly different from pre-exercise, $p<0.01$.

CD11a, CD62L and CD94 expression

γδ T cells were defined by expression of adhesion molecules (CD11a and CD62L) and the NK receptor CD94. CD11a γδ T cells were separated into $CD11a^{high}$ and $CD11a^{low}$ subsets. A significant time effect was evident for changes in $CD11a^{high}$ γδ T cells, with *post hoc* analysis revealing a significant decrease from pre-exercise to 1 h, 2 h, and 4 h post-exercise ($p<0.01$) (Figure 4.8). CD11a expression on γδ T cells are also presented in "low" and "high" concentration groups according to total γδ T cells resting concentration. The "low" and "high" groups decreased by 5.6% and 18.4% ($CD11a^{low}$), 38.6% and 45.5% ($CD11a^{high}$), respectively to nadir values. In examining pre-exercise concentration versus the difference between pre-exercise and nadir values, there was a significant relationship in $CD11a^{high}$ (r = -0.91, $p<0.001$), but not in $CD11a^{low}$ (r = -0.30, $p=0.41$).

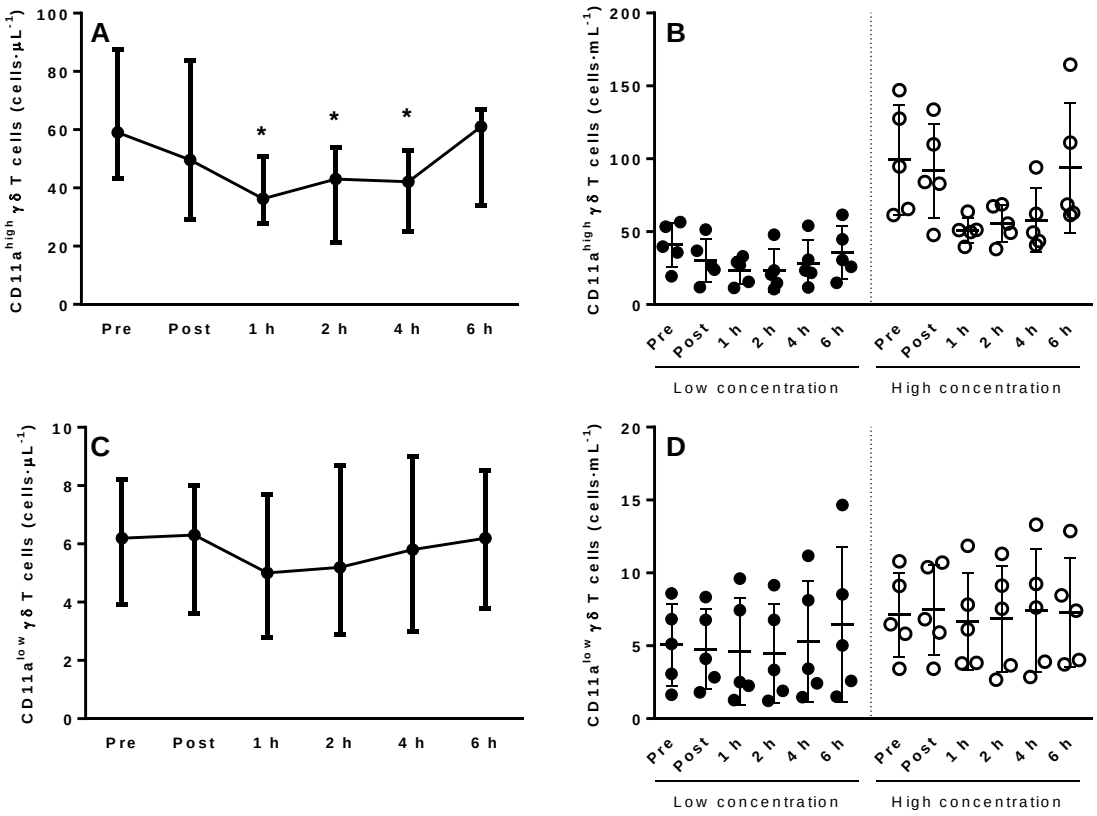

Figure 4.8. High (A) and low (C) expression of CD11a on γδ T cells, and individual concentrations of these subsets divided into "low" and "high" concentration groups (B and D) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). All data non-normally distributed. *, significantly different from pre-exercise, $p<0.01$.

CD62L expression reflects lymph node-migratory potential, and can be divided into CD62L^{high} and CD62L^{low} γδ T subsets. There was a significant time effect evident for changes in the both subsets. *Post hoc* analysis revealed that CD62L^{high} γδ T cell concentration was significantly lower than pre-exercise values at 1 h, 2 h, and 4 h post-exercise, while CD62L$^{low/-}$ γδ T cell concentration was significantly lower than pre-exercise values at 1 h post-exercise (p<0.01) (Figure 4.9). Data for expression of CD62L on γδ T cells are also presented in "low" and "high" concentration groups according to the resting concentration of total γδ T cells. The "low" and "high" concentration groups decreased by 38.1% and 44.8% (CD62L^{low}), and 37.5% and 44.8% (CD62L^{high}), respectively from pre-exercise to nadir values. When examining pre-exercise concentration versus the difference between pre-exercise and nadir values, there was a significant positive relationship in CD62L^{low} (r = 0.95, p<0.001), and CD62L^{high} (r = 0.82, p<0.01).

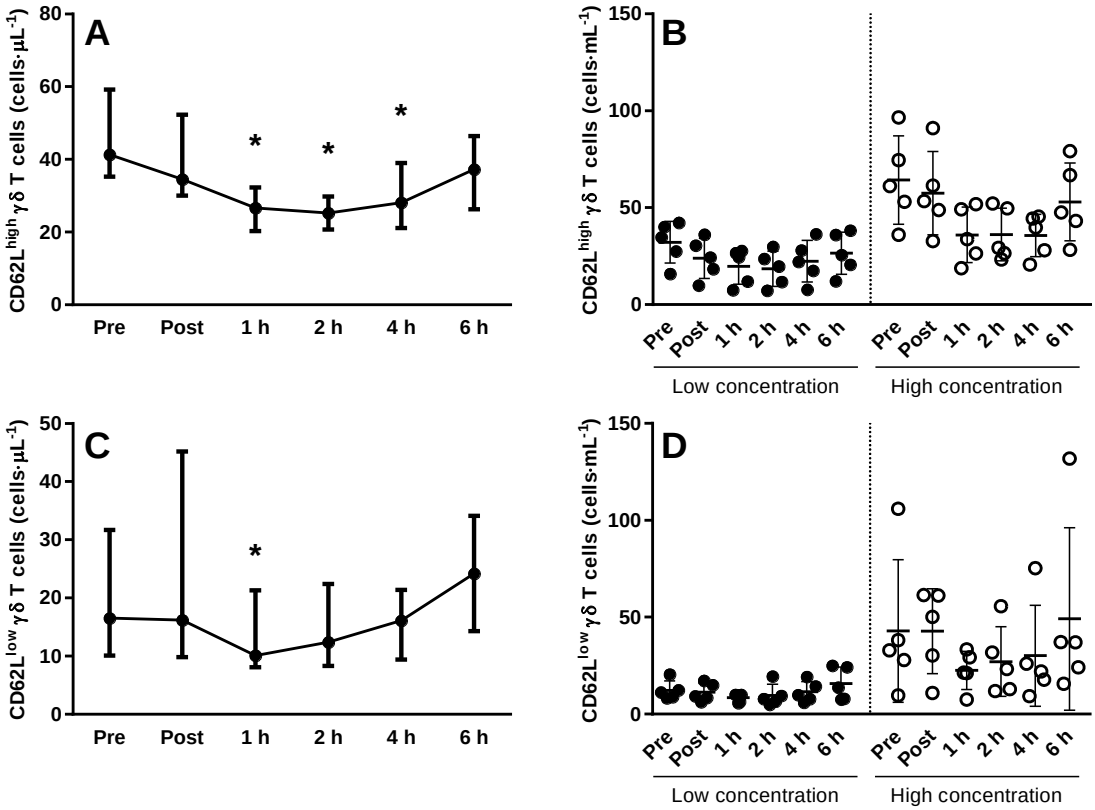

Figure 4.9. High (A) and low (C) expression of CD62L on γδ T cells, and individual concentrations of these subsets divided into "low" and "high" concentration groups (B and D) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). All data non-normally distributed. *, significantly different from pre-exercise, $p<0.01$.

CD94 γδ T cells were defined as $CD94^+$ and $CD94^-$ in relation to innate recognition of aberrant MHC expression on infected and cancerous cells. A significant time effect was evident in both $CD94^+$ and $CD94^-$ cells. *Post hoc* analysis revealed that both $CD94^+$ and $CD94^-$ γδ T cell concentrations were significantly lower than pre-exercise values at 1 h, 2 h, and 4 h post-exercise ($p<0.01$) (Figure 4.10). Data for expression of CD94 on γδ T cells are also presented in "low" and "high" concentration groups according to the pre-exercise concentration of total γδ T cells. The "low" and "high" concentration groups decreased by 10.5% and 39.0% ($CD94^+$), and 49.5% and 52.5% ($CD94^-$), respectively from pre-exercise to nadir values. When examining the relationship between pre-exercise concentration and the difference between pre-exercise and nadir values, there was a significant relationship in $CD94^+$ ($r = -0.84$, $p<0.01$), and $CD94^-$ ($r = -0.93$, $p<0.001$).

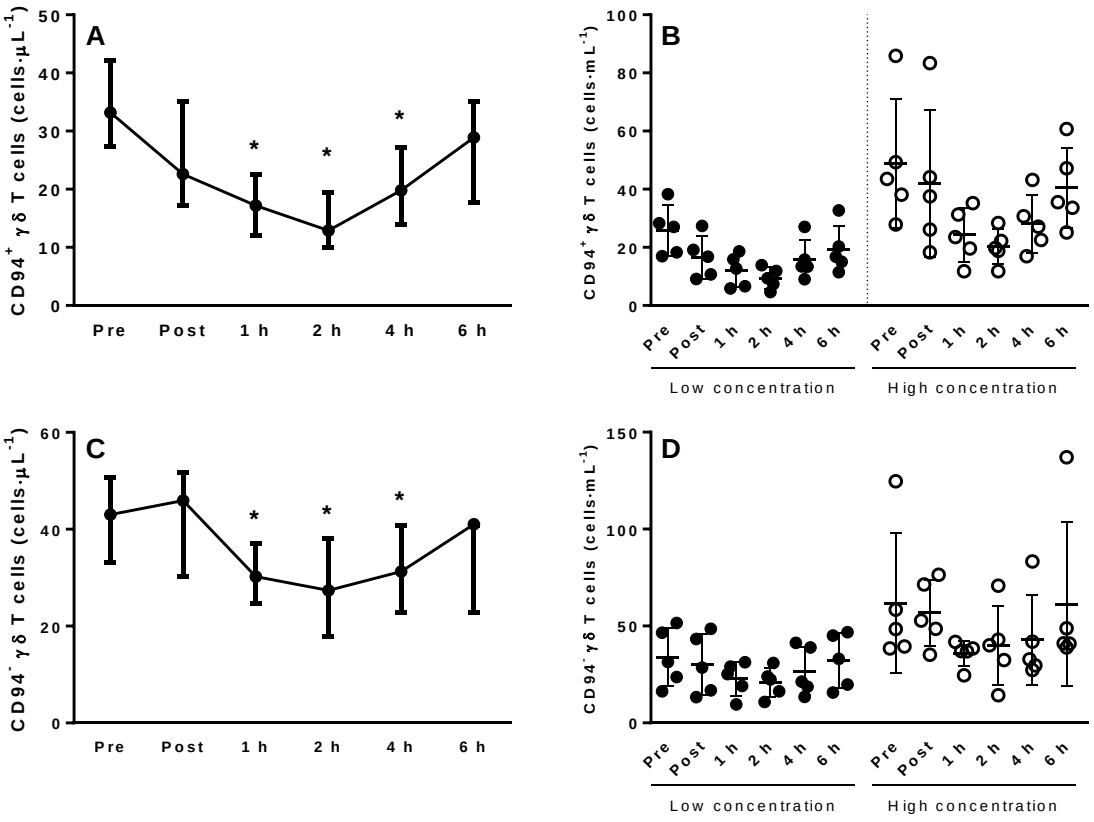

Figure 4.10. Positive (A) and negative (C) expression of CD94 on γδ T cells, and individual concentrations of these subsets divided into "low" and "high" concentration groups (B and D) before and after a bout of prolonged heavy-intensity exercise. Data represent median (1st and 3rd quartile). All data non-normally distributed. *, significantly different from pre-exercise, $p<0.01$.

Dendritic cell phenotypes

Exercise stimulated changes in total dendritic cells and cell phenotypes. A significant time effect was evident for changes in tDCs (Figure 4.11), mDCs (Figure 4.12), pDCs (Figure 4.13) and $CD14^{-/low}CD16^{+}$ DCs (Figure 4.14). *Post hoc* analysis revealed that mDC and $CD14^{-/low}CD16^{+}$ DC concentrations were significantly lower than pre-exercise values at 1 and 2 h post-exercise ($p<0.01$). Data for total DCs, and its phenotypes are also presented in "low" and "high" concentration groups according to the resting concentration of total DCs. The "low" and "high" concentration groups decreased by 31.7% and 29.3% (tDCs), 21.7% and 35.4% (pDCs), 33.1% and 25.4% (mDCs), and 54.4% and 61.2% ($CD16^{+}$DCs), respectively from pre-exercise to nadir values. When examining pre-exercise concentration

versus the difference between pre-exercise and nadir values, there was a significant positive relationship in tDCs (r = -0.64, $p<0.05$), pDCs (r = -0.85, $p<0.001$), and $CD16^{+}$DCs (r = -0.93, $p<0.001$), but not in mDCs (r = -0.55, $p=0.1$).

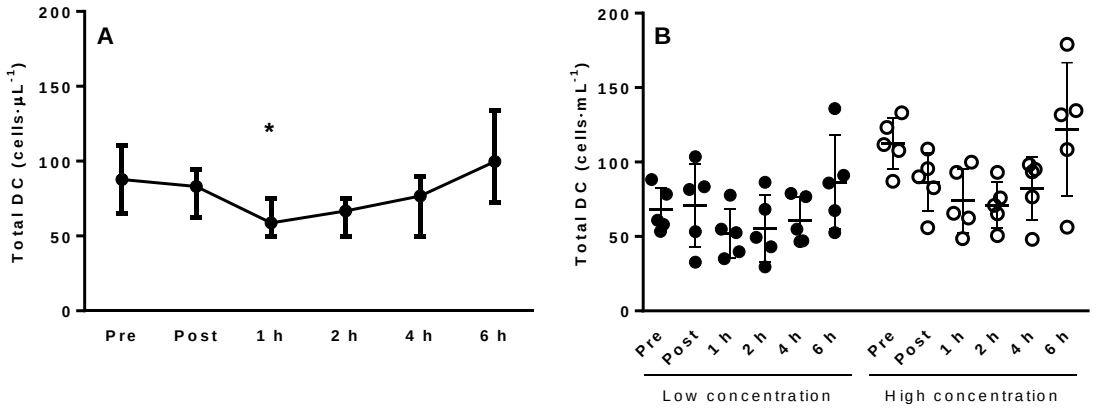

Figure 4.11. Total dendritic cell concentration (A), and individual concentrations divided into "low" and "high" concentration groups (B) before and after endurance exercise. Data (A) represent median (1st and 3rd quartile). *, significantly different from pre-exercise $p<0.01$.

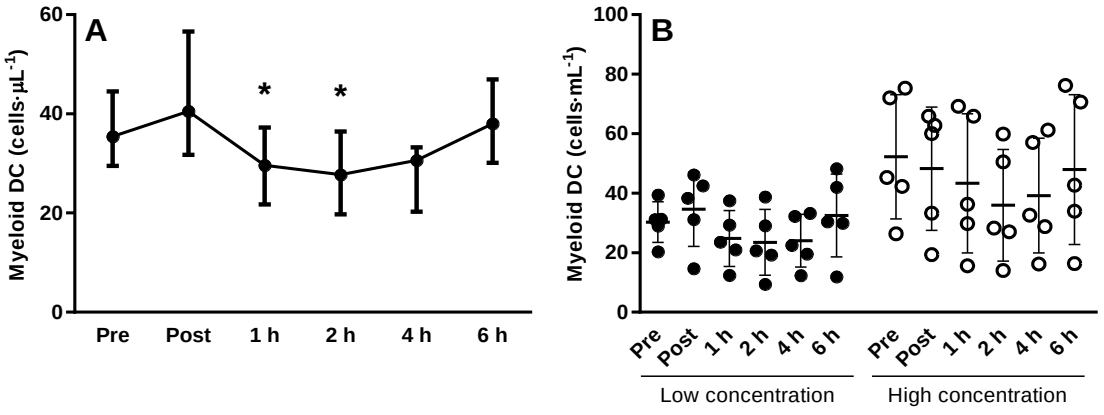

Figure 4.12. Myeloid dendritic cell concentration (A), and individual concentrations divided into "low" and "high" concentration groups (B) before and after endurance exercise. Data (A) represent median (1st and 3rd quartile). *, significantly different from pre-exercise $p<0.01$.

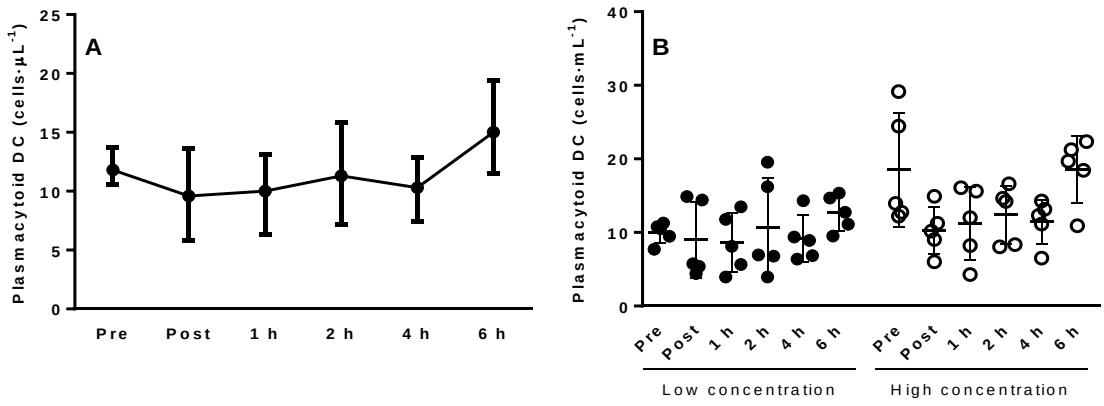

Figure 4.13. Plasmacytoid dendritic cell concentration (A), and individual concentrations divided into "low" and "high" concentration groups (B) before and after endurance exercise. Data (A) represent median (1st and 3rd quartile). *, significantly different from pre-exercise $p<0.01$.

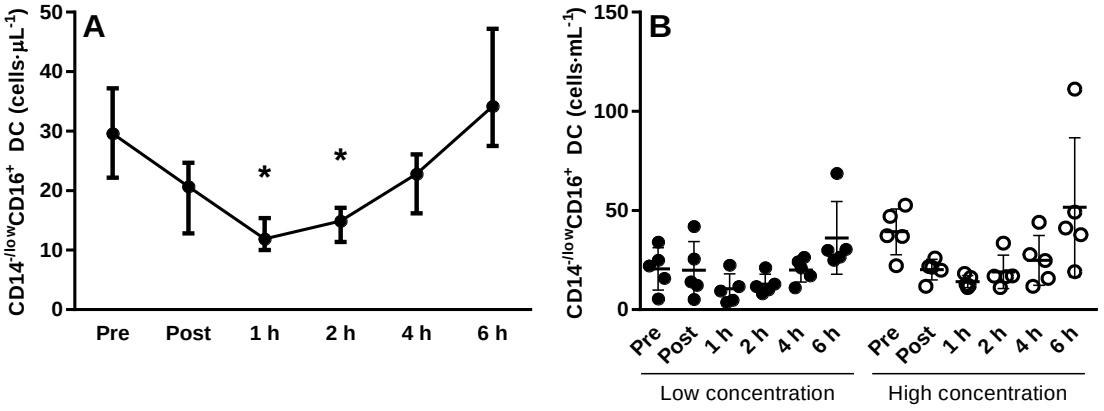

Figure 4.14. $CD14^{-/low}CD16^{+}$ dendritic cell concentration (A), and individual concentrations divided into "low" and "high" concentration groups (B) before and after endurance exercise. Data (A) represent median (1st and 3rd quartile). *, significantly different from pre-exercise $p<0.01$.

Discussion

The present study examined changes in γδ T cell and dendritic cell subsets in peripheral blood in response to prolonged heavy-intensity exercise. Results revealed a significant decrease in the concentration of γδ T cells, due to redistribution of the δ2 subset that persisted up to 4 h post-exercise. Specifically, naïve ($CD45RA^{+}/CD27^{+}$), and central memory ($CD45RA^{-}/CD27^{+}$) γδ T cells were predominantly responsible for the decrease in the δ2 subset. Furthermore, data was divided into "high" and "low" concentration groups. This provided information on the specific cell types mobilised during exercise-induced stress, and the influence of resting cell concentration on the cellular redistribution. These data highlight the transient, biphasic change in circulating γδ T cells following acute exercise. A significant time effect was also evident in some cell types (e.g., total lymphocytes, δ2 EM, pDCs), changes in the concentrations of these cells were not statistically significant ($p<0.01$) at any specific time point following exercise.

The decrease in γδ T cell concentration occurred despite no significant changes in total lymphocyte concentration. This phenomenon suggests aggressive egress of γδ T cells out of the circulation, compared with T and B cells. The absence of any significant change in lymphocyte concentration differs from some previous research [1, 107, 315]. However, in response to a 2.5-h treadmill run at 75 % of maximal oxygen uptake, there was no significant change in lymphocyte concentration immediately post-exercise, while midway through the exercise bout, a significant increase occurred [155]. This may reflect the duration of the bout and the well trained status of the individuals recruited. As part of the immunological response to stress, ingress of lymphocytes into the circulation occurs followed by egress out of the blood to target organs such as the skin and lung, or sites of immune activation, or back to compartments from which the cells were originally mobilised (e.g., the marginated pool, spleen, and bone marrow) [106]. Egress of γδ T cells out of circulation in the present study contrasts with the ingress of γδ T cells into circulation previously observed following acute psychological stress, 20-min bouts of low- and high-intensity exercise, and β-agonist infusion [104]. This difference in redistribution of γδ T cells between the present study and that of Anane et al. likely reflects differences in the types of stress, the elapsed time between initial activation of the stress response, and subsequent timing of blood samples [156].

While the δ1 subset remained unchanged, the δ2 subset decreased significantly in concentration during the post-exercise period (Figure 4.4, 4.5). This difference may reflect differences in the functions of the two subsets. The δ2 subset accounts for the majority of γδ T cells in circulation [79]. These cells function importantly in the role of TCR-dependent activation by phosphoantigens, enabling them to respond to a large range of pathogens [79]. Conversely, the δ1 subset is predominantly found in the mucosa, the skin and intestines, representing only a minor proportion of γδ T cells in peripheral circulation. These δ1 γδ T cells exert important functions in local epithelial immune surveillance, and assist in local wound healing [316]. Different changes in these subsets have also been reported following psychological stress, when there was a trend towards greater ingress of δ2 into circulation [163]. Caution is advised when comparing cell ingress versus cell egress, because the magnitude of initial cell ingress does not always reflect the degree of delayed cell egress [106]. Naïve and central memory γδ T cells were largely responsible for the decrease in the δ2 subset concentration that we observed after exercise. It would seem at odds that naïve and central memory phenotypes are preferentially redistributed, when both effector memory phenotypes possess immediate effector functions [317]. Perhaps, the ability for rapid cell expansion in response to antigen or cytokine stimulation provides a distinct innate advantage compared to cells with immediate specific effector responses (e.g., effector memory and effector memory RA γδ T cells) [85].

Differentiation of cell phenotype through cell surface antigen expression further highlighted the functional diversity of the γδ T population. A significant decrease was observed in $CD11a^{high}$ γδ T cell concentration up to 4 h post-exercise. This may be expected, because CD11a functions in cellular adhesion, is typically up-regulated on endothelial cells in the proximity of inflamed tissues [92], and is highly sensitive to catecholamine secretion [162]. Additionally, psychological stress triggers selective ingress of γδ T cells that express high levels of CD11a [163]. Both $CD62L^{high}$ and $CD62L^{low}$ γδ T cells decreased significantly following the 2 h cycling bout. $CD62L^{low}$ reached a nadir at 1 h post-, while $CD62L^{high}$ remained significantly below pre-exercise concentration until 4 h post-exercise. The discrepancy between ‘high’ and ‘low’ CD62L expression following

stress has been observed in T helper cells, cytotoxic T cells, NK cells and B cells [106, 318], and likely reflects the successive decrease in CD62L expression with the progressive increase in phenotype maturity [79, 86]. Finally, both $CD94^{+}$ and $CD94^{-}$ γδ T cell concentrations were significantly lower than pre-exercise values up to 4 h post-exercise. Both subsets are mobilised following psychological stress [163], and it appears there is no preference as to cytotoxic capability when undergoing egress following exercise-induced stress.

Total dendritic cell concentration significantly decreased from pre-exercise to 1 h post-exercise. This was highlighted by a significant decrease in mDCs and $CD14^{-/low}CD16^{+}$ DCs up to 2 h post-exercise. Because dendritic cells undergo a maturation process after entering the tissue [63], the effects of stress-induced redistribution may have important implications for immune surveillance in the periphery [319]. Previous research has reported that dendritic cell concentration increases acutely following surgery, or a treadmill exercise test to exhaustion, with an equal rise in mDCs and pDCs [105]. Furthermore, immediately following a marathon race, mDC concentration increases, while pDC concentration decreases [164]. Collectively, these results may reflect cellular redistribution as observed following acute stress in mice [319]. The difference in egress between mDCs and pDCs reflects specific functions of these cell types. mDCs are characteristically first responders to pathogens and other causes of inflammation and are able to stimulate allogen- and antigen-specific T cell responses [74]. The ability of these cells to respond to stress and be redistributed to areas of infection is likely important and reflective of the results in the present study. Additionally, mDCs are reported to mainly trigger a Th1-immune response [320], whereas pDCs trigger a Th2-immune response [321]. The decrease in Th1-related mDCs and no change in Th2-related pDCs is a similar response to changes in Th1 and Th2 cells in circulation following a 2.5-h treadmill run, where type 1 cells decreased while type 2 cells remained unchanged 2 h after exercise [155]. Similar to mDCs, $CD16^{+}$ DCs are involved in T cell activation through allogeneic stimulation and type 1 cytokine responses [65, 322]. The result of the present study confirms that mDCs and $CD16^{+}$ DCs also share similar characteristics of cellular redistribution following exercise-induced stress. Together, these results demonstrate an exercise-induced redistribution of dendritic cells,

which features selective egress of those cells with greater ability to stimulate Th1 responses.

In addition to conventional analysis, individuals were divided into “high” vs “low” concentration groups according to circulating cell concentration at rest. The concept of sorting subjects into “high” and “low” and/or “non” responder groups has been used in research previously, such as when examining the effectiveness of a training program or intervention on individuals, either healthy populations [323, 324], diseased populations [325, 326], or the highly trained [327]. The concept in the present study is slightly different, rather examining the influence of resting cell concentration in peripheral circulation on cellular ingress (granulocytes and monocytes) and egress (lymphocytes, γδ T cells, and dendritic cells). This is of interest, as i) an acute stressor can cause divergent responses in the severity of leucocyte redistribution between individuals, and ii) low resting concentrations of cell subsets have been of interest in athletic populations previously [328, 329]. A number of interesting observations were identified. Subjects were sub-divided into “high” and “low” concentration groups on the basis of the resting concentrations of granulocytes, monocytes, lymphocytes, γδ T cells (δ1 and δ2 subsets, and CD11a, CD62L, and CD94), and tDCs (mDCs, pDCs, and $CD16^+$ DCs). Although statistical analysis did not uncover any significant difference between the “high” and “low” concentration groups (n = 5 per group), a number of interesting observations were detected. While some subsets appeared to have quite a uniform response between “low” and “high” regardless of initial resting concentration, others demonstrated a much more divergent response. Concerning cellular ingress; granulocytes and monocytes appeared to feature similar changes in cell concentration from rest to peak values, regardless of initial concentration. Furthermore, Pearson’s correlation coefficients were $r = 0.16$ and $r = 0.02$ for granulocytes and monocytes, respectively suggesting a small to no correlation between resting and peak concentration. For cellular ingress, results varied between cell types. For lymphocytes, total γδ T cells, δ1, δ2, CD62L (both low and high subsets), CD11a (both low and high subsets), CD94 (both positive and negative subsets), mDCs, and $CD16^+$ DCs the “low” resting concentration group resulted in a lower percentage concentration change to nadir compared to the “high” resting concentration group. Conversely, tDCs and pDCs

saw a higher percentage change in the "low" resting concentration group. Furthermore, there was a significant large (0.7 to 1.0) negative correlation between resting and the decrease to nadir concentrations for lymphocytes, total γδ T cells, δ1, δ2, CD62L (both low and high subsets), $CD11a^{high}$ (not low), CD94 (both positive and negative subsets), mDCs, and pDCs. For these subsets, it appeared that those individuals with a higher resting concentration pre-exercise experienced a greater drop to nadir values during the post-exercise period, whereas those individuals with a lower resting cell concentration experienced less of a drop, and in some cases, an increase in cell concentration. Potentially, the magnitude of egress in relation to resting leucocyte concentration may have implications for the effectiveness of cellular redistribution following the stress response. It is recognised in previous research that elite athletes experience leucopenia at rest driven primarily by neutropenia [328, 329]. Furthermore, a high-intensity training block resulted in impaired redistribution of $CD8^+$ T lymphocytes in elite cyclists following exercise [330]. Questions remain wether this is due to decreased resting concentration of leucocytes or a more efficient stress response mounted by the athlete due to regularly experiencing such efforts. A previous study has suggested that it may be the latter [329], however this research was examining resting concentrations rather than in response to an acute stressor. The present study has provided more evidence to suggest resting peripheral cell circulating and cellular redistribution are closely linked phenomenon.

Finally, the present study decided not to include a 24 h post-exercise time point. It was determined that the 24-h sample was the least important sample, compared to sampling closer to the end of the bout of exercise. While countless studies have included time points at immediately post-, 1 h post-, and 24 hours post-exercise [104, 105, 150, 153, 155, 159, 163, 165, 308, 331], very few have examined the period between immediately post- and 6 h post-exercise [1, 195-197, 332]. This time period was considered more important, both within the context of the 'Open Window' Theory [44], and when considering athletes completing multiple bouts of exercise on the same day. Furthermore, many studies, including my Honours research highlighted most immune subsets had returned to resting level 24 hours post-exercise, even following repeated exercise [1, 150, 155, 177-179, 187,

189], this has also been highlighted in a recent position statement review article [111]. Thus more focus was placed on time points from immediately post-, to 6 h post exercise.

In summary, the present study provides novel and detailed insights into the time course of changes in γδ T cell subsets and dendritic cells during recovery from prolonged, intense physical stress. It has been proposed that an 'open window' transiently exists in the hours after exercise, whereby individuals are potentially more susceptible to contracting upper respiratory illnesses [107]. In the present study, the circulating concentration of most cell types decreased during the post-exercise period. Rather than reflecting a decline in immunocompetence, it is more likely that this response reflects a change in immunosurveillance, whereby cells move selectively to regions of the body where they may encounter pathogens (e.g., skin, lungs and gastrointestinal tract) [333]. Alternatively, some T cells may also be redistributed to bone marrow, where they could play a role in regulating the production of granulocytes [333]. Future research could investigate whether alterations in the distribution of γδ T cell subsets and dendritic cells in blood following exercise is associated with changes in their capacity to respond to infectious challenges, and changes in cellular function.

5. STUDY IIIA – THE EFFECT OF REPEATED PROLONGED HEAVY EXERCISE ON T HELPER CYTOKINE PROFILES

Abstract

The purpose of this study was to investigate the effects of prior heavy-intensity exercise on whole blood T helper cytokine profiles during recovery from a second bout of exercise. Ten well-trained endurance cyclists (mean ± SD: age 23.2 ± 3.2 years; height 1.82 ± 0.06 m; body mass 74.8 ± 6.2 kg; peak oxygen uptake 59.0 ± 4.3 $mL.kg^{-1}.min^{-1}$) performed two exercise trials on different occasions. On the first occasion, they cycled in the morning and afternoon for 1.5 h at 90% of their second ventilatory threshold, with 4 h separating the two bouts. On the second occasion, they cycled for 1.5 h at the same intensity, but only in the afternoon. Blood samples were collected throughout the recovery period post-exercise. Whole blood was stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin, and a cytometric bead array was used to quantify Th1/Th2/Th17 cytokine production. There were significant increases in cytokine production from pre-exercise of Th1 cytokine; IL-2, and Th2 cytokines; IL-4 and IL-10. When adjusted according to total leucocyte concentration at each time point, a significant increase from pre-exercise was observed in Th1 cytokines; IL-2, TNF, and IFN-γ, and Th2 cytokines; IL-4 and IL-10. However, there was no significant difference between the single and repeat exercise bout trials at any time point. Importantly, a significant time x trial effect was present in IL-17A production, suggesting a potential effect of repeated exercise on the ability to produce IL-17A which may be important considering its role in rapid response to infection.

Introduction

Training of endurance athletes typically features repeated exercise bouts of heavy-intensity and prolonged duration. Such exercise results in transient changes in circulating leucocyte concentrations, and potentially changes in immune function and an increased risk of symptoms associated with upper respiratory illnesses (URIs) [1-5]. While acute stress (such as regular exercise) exhibits positive effects on the immune system, chronic stress is considered harmful, increasing the occurrence of opportunistic infections [102, 108]. During a typical exercise training schedule, endurance athletes regularly activate the stress response, sometimes without adequate recovery between stressors. This regular stress-induced immunological challenge can alter the balance between a healthy immune system and training-induced immunosuppression [34, 36, 334]. The reported increased incidence of symptoms associated with URIs in elite athletes regularly occurs during periods of heavy training and competition [3-5, 24, 32, 335]. It has been proposed that repeated exercise without sufficient recovery may suppress immune function, which may in turn increase susceptibility to URIs [76, 108, 336].

Some previous research has examined immune system function following successive exercise bouts [156, 177-188]. Of these studies, a number have examined immune variables unrelated to plasma cytokines [156, 177-185, 187, 188]. Furthermore, while these studies do examine repeated exercise, the duration of the exercise and/or the recovery period or the intensity of the exercise is not comparable to the current study [180-185]. Of the previous studies, a number of these have highlighted a ‘carry-over’ or ‘additive’ effect of repeated exercise bouts on lymphocyte and neutrophil concentration changes [177-179, 186-188]. This ‘additive’ effect of repeated exercise in combination with inadequate recovery has been hypothesised to lead to chronic suppression of immune function [76]. Two studies have examined plasma cytokine concentrations, IL-6 and IL-1ra with repeated exercise [186, 189]. While one study highlighted this ‘carry-over’ effect [189], the other did not find any significant difference between single and repeat exercise protocols [186]. Importantly, these studies examined plasma cytokine concentrations, whereas no previous study has examined cytokine production following mitogen stimulation.

Mitogen stimulation is used for *in vitro* assessment of cellular immunity for research purposes, in addition to examining patients with immunodeficiencies [231], autoimmunity [232], infectious diseases [233], and cancer [234], and is thought to reflect immune function *in vivo*. Stimulating isolated leucocytes allows a functional representation of a cellular population at rest, or while under pre-determined controlled conditions. A common cell stimulation cocktail used in immunology research combines phorbol 12-myristate 13-acetate (PMA) and ionomycin [235]. Treatment with PMA and ionomycin is sufficient to induce activation of many cell types to produce cytokines [236, 257]. This cocktail can be used to induce cytokine production in *in vitro* cell culture for subsequent detection in immunoassays, either by ELISA, cytometric bead array [258], or intracellular cytokine identification [155]. PMA is a small organic compound which diffuses through the cell membrane into the cytoplasm, where it directly activates protein kinase C (PKC). Activation of PKC triggers calcium ion release and mobilisation, resulting in a cascade of additional cellular responses mediating T-cell activation [259]. Ionomycin, a calcium ionophore produced by the bacterium *Streptomyces conglobatus*, is used in addition to PMA to trigger calcium release, which is required for nuclear factor of activated T-cell (NFAT) signalling [260]. Stimulation with these compounds bypasses the TCR complex and will lead to activation of several intracellular signalling pathways [261]. This activation results in a rapid up-regulation of cytokine production. Previously, the combined stimulation with PMA and ionomycin was used in analysis of intracellular cytokines following a 2.5 h bout of cycling [155, 159], and a 1.5 h bout of running [159]. Both studies demonstrated exercise-induced changes in type 1 cytokine-producing T cells.

Exercise-induced stress alters glucocorticoid and catecholamine concentrations. Importantly, these stress hormones influence T helper cell function, altering cytokine release, principally by stimulating glucocorticoid and β2 adrenergic receptors, respectively [166]. In turn, these stress hormones influence the activity of cellular (Th1), humoral (Th2), and mucosal (Th17) branches of the immune response [166, 167]. Specifically, cortisol, epinephrine and norepinephrine may suppress cytokine release from antigen presenting cells and Th1 cells, while up-regulating cytokine production from Th2 cells, thereby causing a shift toward Th2 cytokine production [155, 168]. The balance between

Th1 and Th2 immunological responses is very important in maintaining optimum immune health. Furthermore, Th17 cells play a key role in mucosal host defence of the upper respiratory and gastrointestinal tracts against a myriad of airborne antigens [297-299]. Functioning as part of the "first line of defence" in the mucosa, Th17 cytokines have been linked to increased polymeric immunoglobulin receptor (IgR) expression, saliva sIgA and human β-defensin 2 release [300-303]. The effect of repeat exercise may alter this relationship. Previous research has highlighted a 'compounding'effect of repeated exercise on circulating epinephrine, norepinephrine and cortisol concentrations [177]. However, it is unknown how the T helper system would be affected by this increasing response. Previous research has examined changes in the balance of Th1 and Th2 cytokines in plasma in the first 1–2 h after a single prolonged exercise bout [295]. However, no research has examined changes in T helper cytokine secretion (including Th17 cytokines) following repeat bouts of prolonged heavy-intensity exercise.

Mitogen stimulation is an effective method of analysing immune function. Very little is known about the mitogen-stimulated T helper cytokine response to exercise, especially the influence of repeated exercise with limited recovery. The present study examined the effect of repeated prolonged heavy exercise on plasma cortisol concentration, and Th1, Th2, and Th17 cytokine release.

Methods

Subjects

Ten highly-trained male cyclists volunteered to participate in the present study. Physical characteristics of the cyclists are presented in Table 5.1. Subjects were excluded if they exhibited any cardiovascular, metabolic, neurological, or autoimmune disorders. The cyclists provided written informed consent for participation in the study, which was approved by the Bond University Human Research Ethics Committee.

Table 5.1. Physical characteristics, aerobic capacity, and threshold performance data

Subject Characteristics (n=10)	Mean ± SD
Age (years)	23.2 ± 3.2
Height (m)	1.82 ± 0.06
Body mass (kg)	74.8 ± 6.2
BMI ($kg.m^{-2}$)	22.5 ± 1.6
Absolute $\dot{V}O_2$peak ($L.min^{-1}$)	4.4 ± 0.3
Relative $\dot{V}O_2$peak ($mL.kg^{-1}.min^{-1}$)	59.0 ± 4.3
Peak Power (W)	411 ± 28
Peak Power-to-weight (W/kg)	5.5 ± 0.5
VT_1 Power (W)	287 ± 23
VT_1 Power-to-weight (W/kg)	3.8 ± 0.3
VT_2 Power (W)	342 ± 30
VT_2 Power-to-weight (W/kg)	4.6 ± 0.4
Training (km/week	316 ± 75

Data are mean ± standard deviation. BMI: body mass index. $\dot{V}O_2$peak: peak oxygen uptake. VT_1: first ventilator threshold. VT_2: second ventilator threshold. Training: volume in kilometres performed during training per week.

Experimental Design

Each cyclist visited the laboratory on four occasions; for a screening/familiarization session, a maximal exercise test, and two experimental exercise sessions. The experimental exercise sessions (Figure 5.1) involved each cyclist completing both a morning (A.M.-Ex) and afternoon (P.M.-Ex) bout of exercise (Repeat Trial - REP), and on a separate occasion,

only an afternoon bout of exercise (Single Trial - SIN). The two experimental trials were separated by a minimum of seven days, during which time the cyclists were permitted to train as normal. Participants completed the SIN and REP trial in a counter-balanced order. Throughout the testing period, cyclists were required to report any upper respiratory or gastrointestinal symptoms. All cyclists refrained from exercise for 48 h prior to both the maximal exercise test and the experimental trials. Alcohol or caffeine consumption was avoided during the exercise and post-exercise sample period.

Peak oxygen consumption ($\dot{V}O_2$peak) was determined during a graded exercise test in which the participants cycled at their preferred cadence within the range of 70–100 rev.min^{-1}. The test was performed on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The cyclists commenced the graded exercise test at 90 W for 4 min, after which the work rate increased by 30 W/min until volitional fatigue and/or when the cyclists could not maintain their cadence above 70 rev.min^{-1} despite strong verbal encouragement. The criteria used to determine maximal effort at the end of a test were: a plateau in $\dot{V}O_2$ (defined as an increase in $\dot{V}O_2$ values of less than 2 mL.kg^{-1}.min^{-1} between two consecutive 1-min periods in the final stages of the test), a maximal respiratory exchange ratio $\geq$ 1.1 and/or a maximal heart rate within 5% of their individual age-predicted maximum (220-age) [304]. Each cyclist's first (VT_1) and second (VT_2) ventilatory thresholds were determined from the gas-exchange data collected during incremental exercise (ParvoMedics' TrueOne® 2400, Utah, USA), using the ventilatory equivalents methods [267].

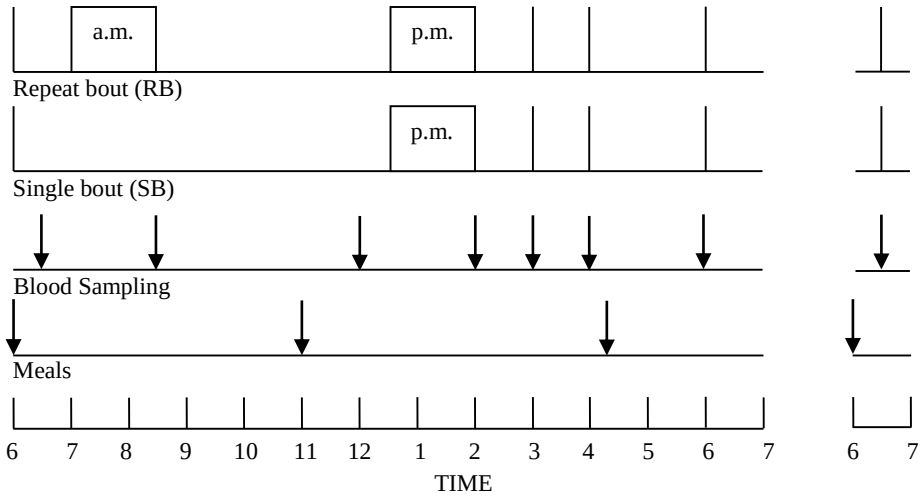

Figure 5.1. Exercise, blood collection, and meal schedule for the Single and Repeat bout data collection protocols.

On arrival for the SIN or REP trials, the cyclists consumed a standardised breakfast (2,000 kJ) of cereal, milk, and juice. Pre-exercise blood samples were collected from the cyclists following breakfast. On the day of the REP trial, the cyclists exercised for 1.5 h at 90% VT_2 on a stationary cycle ergometer (Monark Ergomedic 828 E, Sweden) beginning at 7:00 a.m. (A.M.-Ex) and again at 12:30 p.m. (P.M.-Ex). For the SIN trial, the cyclists also exercised for 1.5 h at 90% VT_2, but only once, beginning at 12:30 p.m. (P.M.-Ex). Heart rate was monitored continuously throughout exercise. Pulmonary gas exchange was sampled for 4 min at 20-min intervals during the exercise bouts to ensure that the cyclists were consistently exercising at the work rate associated with 90% VT_2, and that no drift in $\dot{V}O_2$ occurred. The cyclists ingested water *ad libitum* during exercise. Immediately after exercise, a venous blood sample was collected. In total, eight blood samples were collected at 6.30 a.m. (rest), 8.30 a.m. (post A.M.-Ex), 12.00 p.m. (pre P.M.-Ex), 2.00 p.m. (post P.M.-Ex), 3.00 p.m. (1-h post P.M.-Ex), 4.00 p.m. (2-h post P.M.-Ex), 6.00 p.m. (4-h post P.M.-Ex) and 6.30 a.m. (24-h post rest) on the subsequent day. A post-exercise snack was provided at 8.45 a.m. and 2.15 p.m. consisting of breakfast cereal, milk and honey (1,500 kJ), while larger meals were provided to the cyclists at 11.30 a.m. and 4.15 p.m. consisting of sandwiches and a milkshake (4,500 kJ).

Flow cytometry & Analysis

Briefly, 5 mL of blood was collected into lithium heparin tubes. Two tubes were prepared per sample, each containing a volume of 1 mL of whole blood and 1 mL of RPMI medium. To the stimulated sample, PMA (25 ng/mL) and ionomycin (1 µg/mL) were added, whereas nothing more was added to the unstimulated sample. All samples were mixed and then incubated for 4 h at 37°C. Following incubation, the supernatant was removed and stored at −80°C for subsequent analysis. Th1, Th2 and Th17 cytokine concentrations were quantified using a commercially available cytometric bead array kit (BD Pharmingen, San Diego, CA) to measure the concentrations of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A from supernatant of stimulated samples. In the absence of stimulation with PMA and ionomycin, concentrations of these cytokines were below the detection limit of the assay. The software program FCAP Array v3.0 (BD Bioscience) was used to produce standard curves, and determine unknown concentrations of all seven cytokines.

Statistics

All data are presented as mean ± standard deviation. All statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, USA). Performance measures (heart rate, cadence, power output) across the three exercise trials were compared using one-way ANOVA with repeated measures. All immunological data were analysed using a 2 (single and repeated exercise bouts) x 8 (times of measurement) repeated measures ANOVA. Data were assessed for the assumptions of normality, homogeneity of variance and sphericity. If the assumption of sphericity was violated, the degrees of freedom were adjusted using the Greenhouse-Geisser correction. The time × trial interaction effect is reported. Where significant effects between trials were found ($p<0.05$), separate paired t-tests with Bonferroni corrections for multiple comparisons were performed between each time point across the two trials (SIN versus REP trial). If significant time effects were found ($p<0.05$), separate paired t-tests with Bonferroni corrections for multiple comparisons were performed to compare the pre-exercise resting sample to each subsequent time point. The association between individual cytokine concentrations, and between SIN and REP cytokine concentrations during recovery were

explored using Pearson's correlation analysis to test for correlations. $P<0.05$ was accepted as significant.

Results

Physiological variables during exercise

Mean power output, heart rate and cycling cadence did not differ between exercise trials (Table 5.2).

Table 5.2. Physiological variables during exercise

	SIN P.M.-Ex	REP A.M.-Ex	REP P.M.-Ex	P value
Mean HR (beats.min^{-1})	151 ± 11	150 ± 11	150 ± 10	0.359
Mean Cadence (revolutions.min^{-1})	87.9 ± 7.7	87.3 ± 7.8	87.7 ± 7.9	0.089
Mean Power Output (W)	297.0 ± 28.5	297.4 ± 27.4	294.5 ± 27.0	0.143

Data are mean ± standard deviation. HR: heart rate. SIN: single exercise trial. REP: repeated exercise trial. P.M-Ex: afternoon exercise bout. A.M.-Ex: morning exercise bout.

Leucocyte concentrations

Difference between trials. Immediately post-A.M.-Ex leucocyte concentration was significantly higher in the REP trial, compared to the SIN trial. The leucocyte concentration of the REP trial continued to be significantly above the SIN trial at pre-P.M.-Ex.

Effect of exercise. When trials were examined individually, all exercise bouts resulted in a significant increase in leucocyte concentration from pre- to post-exercise (p<0.001, Figure 5.2).

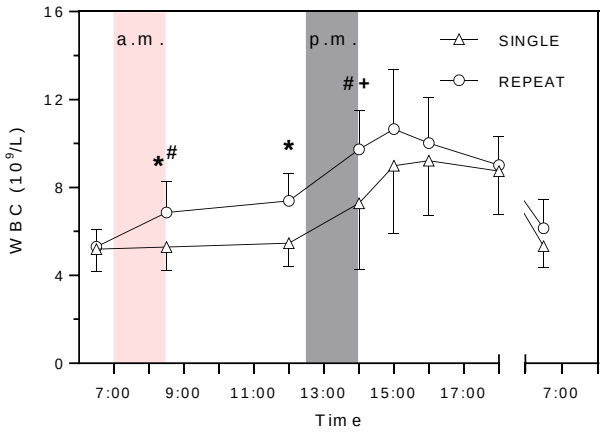

Figure 5.2. Total leucocyte concentration before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. Leucocytes: 0.172; <0.001; 0.001. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, Signifies a significant difference between trials at time point. #, signifies a significant difference from pre- to immediately post-exercise for the REP trial. +, signifies a significant difference from pre- to immediately post-exercise for the SIN trial.

Th 1 cytokines

Difference between trials. There was no significant time x trial interaction or main effect of trials present for production of IL-2, TNF or IFN-γ. There was a trend towards significance in the time x trial interaction of IL-2 concentration (p=0.07).

Effect of exercise. A significant increase was observed in IL-2 concentration from pre-exercise to post-P.M.-Ex in the SIN trial, both in absolute concentration (Figure 5.3.A) and when adjusted according to leucocyte concentration (Figure 5.3.B). Furthermore, a significant increase was observed in TNF concentration from pre-exercise to post-P.M.-Ex in the SIN trial when adjusted according to leucocyte concentration (Figure 5.4.B). Finally, a significant increase was observed in IFN-γ concentration from pre-exercise to post-P.M.-Ex in the SIN trial when adjusted according to total leucocyte concentration (Figure 5.5.B).

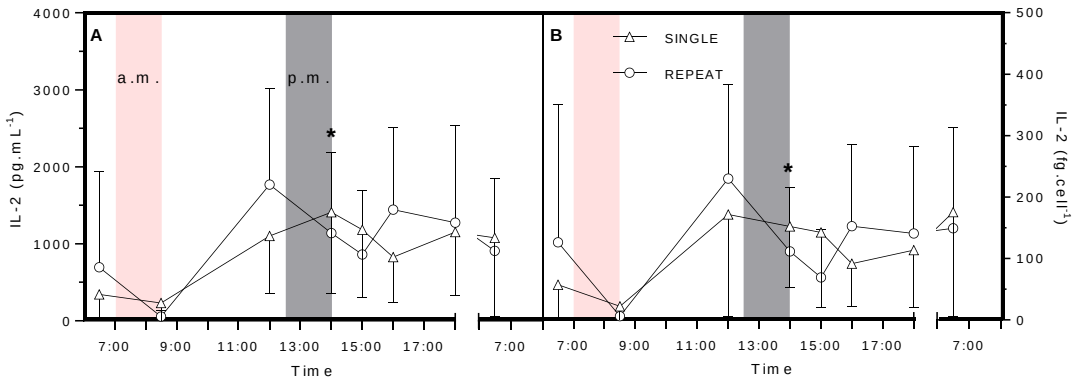

Figure 5.3. IL-2 (Th1) concentration (A) and IL-2 production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.070; 0.011; 0.412. B: 0.261; 0.027; 0.712. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the

main effect of time; the third p value represents the main effect of trial. *, significantly different from pre-exercise in single trial, p<0.01.

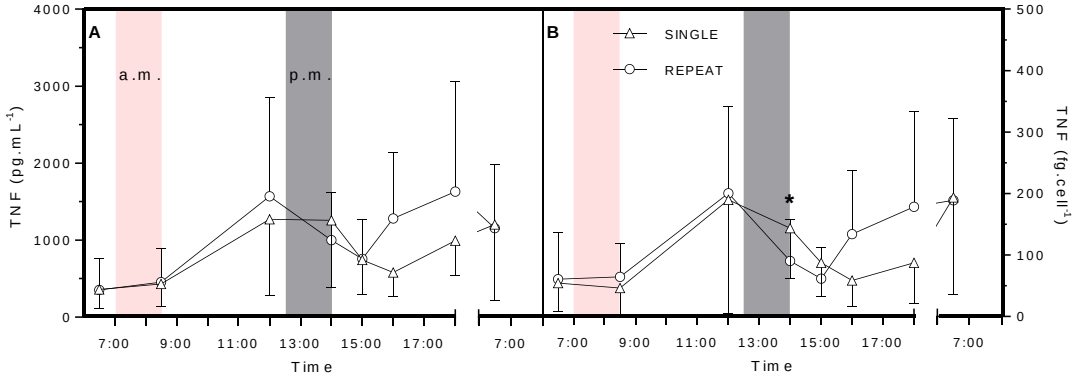

Figure 5.4. TNF (Th1) concentration (A) and TNF production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.154; 0.020; 0.095. B: 0.142; 0.014; 0.297. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, significantly different from pre-exercise in single trial, $p<0.01$.

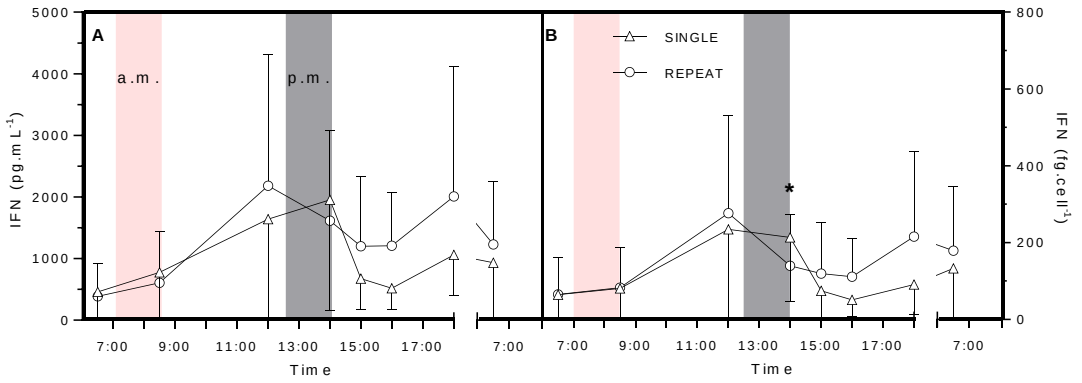

Figure 5.5. IFN-γ (Th1) concentration (A) and IFN-γ production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.676; 0.510; 0.463. B: 0.290; 0.050; 0.091. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, significantly different from pre-exercise in single trial, $p<0.01$.

Th 2 cytokines

Difference between trials. There was no significant time x trial interaction present for production of IL-4, IL-6 or IL-10. There was a trend towards significance in IL-4 concentration (p=0.062).

Effect of exercise. A significant increase was observed in IL-4 concentration from pre-exercise to pre-P.M.-Ex, post-P.M.-Ex, 1 h, 2 h, and 4 h post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex, post-P.M.-Ex, and 2 h post-P.M.-Ex in the REP trial (Figure 5.6.A). When adjusted according to leucocyte concentration a significant increase was observed in IL-4 from pre-exercise to pre-P.M.-Ex, post-P.M.-Ex, 1 h, and 4 h post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex in the REP trial (Figure 5.6.B). No significant time effect was present in IL-6 at any time point. Additionally, a significant increase was observed in IL-10 concentration from pre-exercise to 1 h, and 4 h post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex in the REP trial (Figure 5.8.A). When adjusted according to total leucocyte concentration a significant increase was observed in IL-10 from pre-exercise to post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex in the REP trial (Figure 5.8.B).

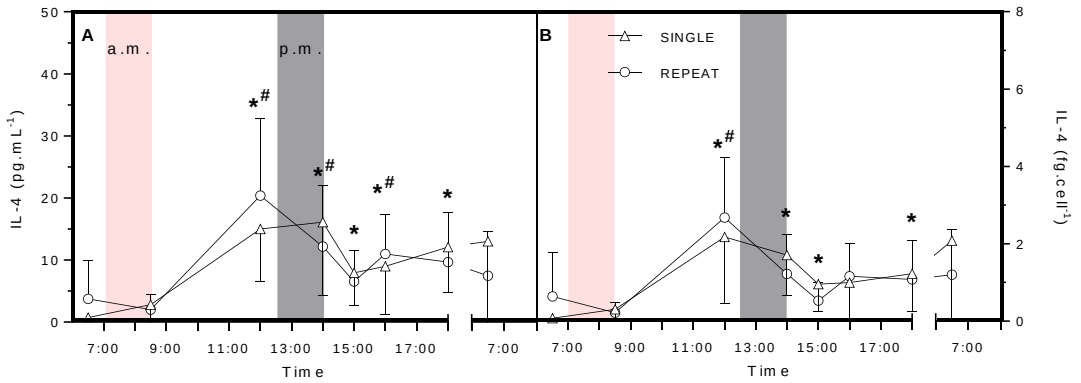

Figure 5.6. IL-4 (Th2) concentration (A) and IL-4 production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.062; <0.001; 0.999. B: 0.125; 0.001; 0.609. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, significantly different from pre-exercise in single trial, p<0.01. #, significantly different from pre-exercise in repeat trial, p<0.01.

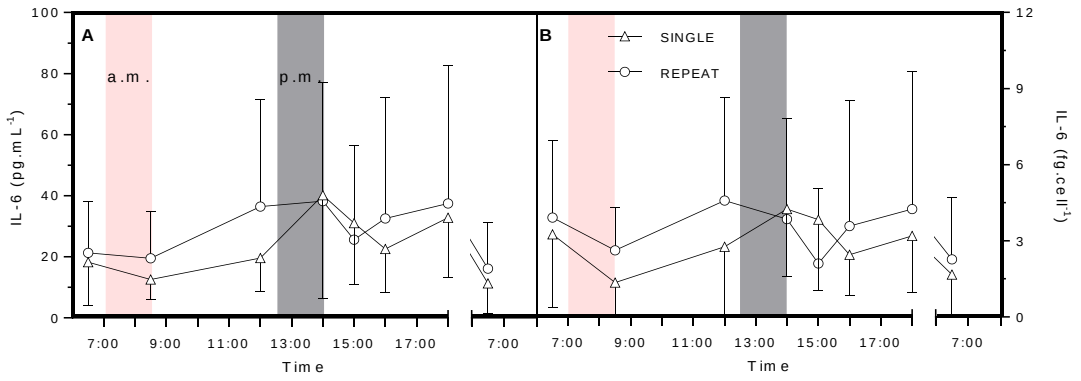

Figure 5.7. IL-6 (Th2) concentration (A) and IL-6 production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.359; 0.294; 0.499. B: 0.472; 0.178; 0.506. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial.

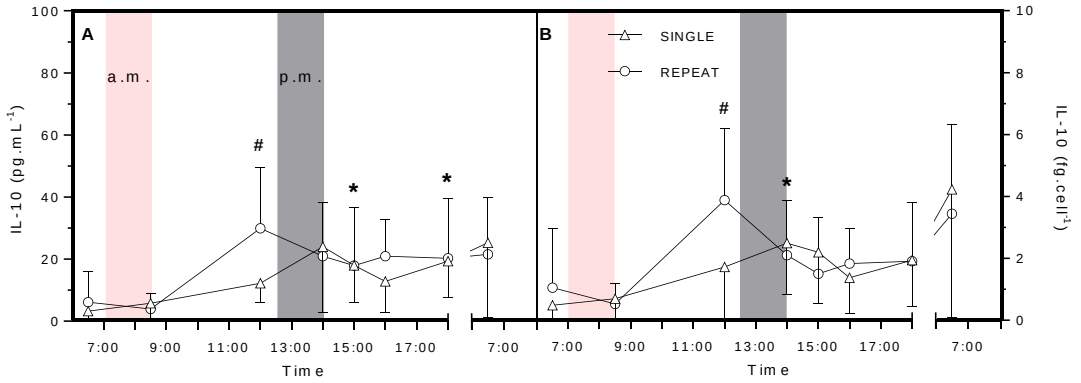

Figure 5.8. IL-10 (Th2) concentration (A) and IL-10 production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.122; 0.004; 0.163. B: 0.125; 0.004; 0.581. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, significantly different from pre-exercise in single trial, $p<0.01$. #, significantly different from pre-exercise in repeat trial, $p<0.01$.

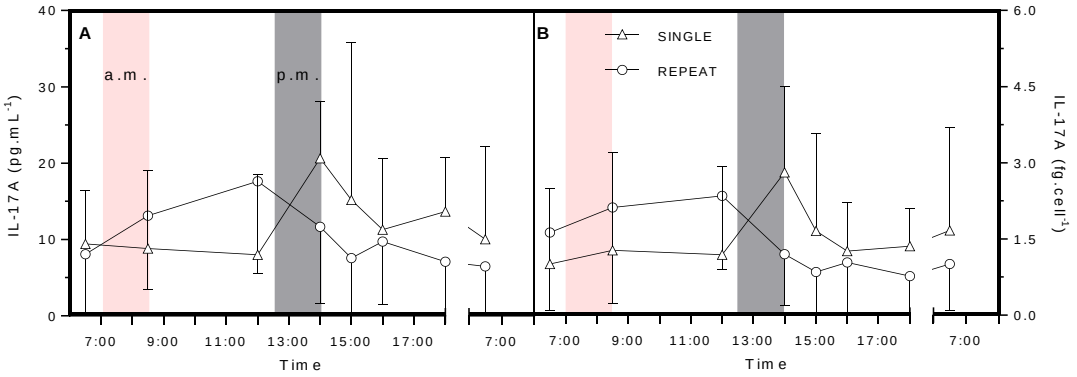

Figure 5.9. IL-17A (Th17) concentration (A) and IL-17A production normalised to WBC concentration (B) before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. A: 0.016; 0.426; 0.811. B: 0.029; 0.439; 0.502. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial.

Th 17 cytokine

Difference between trials. There was a significant time x trial interaction present for both IL-17A concentration (p=0.016) and IL-17A concentration normalised to WBC count (p=0.029).

Correlations between cytokines and trials

As displayed in Table 5.3, individual cytokines were compared via Pearson's correlation coefficients to examine any possible relationships between each cytokine. Significant correlations were observed between all individual cytokines. Additionally, the relationships between SIN and REP cytokine release from post-P.M.-Ex to 24 h post-exercise were examined (Table 5.4). Significant associations were observed for IFN-γ and IL-4.

Table 5.3. Associations between cytokine profiles of each individual cytokine.

	Th1			Th2			Th17
	IL-2	TNF	IFN-γ	IL-4	IL-6	IL-10	IL-17A
IL-2		0.70*	0.51*	0.83*	0.77*	0.82*	0.46*
TNF	0.70*		0.93*	0.67*	0.64*	0.58*	0.42*
IFN	0.51*	0.93*		0.53*	0.47*	0.34*	0.35*
IL-4	0.83*	0.67*	0.53*		0.64*	0.75*	0.41*
IL-6	0.77*	0.64*	0.47*	0.64*		0.79*	0.40*
IL-10	0.82*	0.58*	0.34*	0.75*	0.79*		0.38*
IL-17A	0.46*	0.42*	0.35*	0.41*	0.40*	0.38*	

* $p < 0.001$.

Table 5.4. Associations between SIN and REP bouts for each individual cytokine following PM-Ex.

	Th1			Th2			Th17
	IL-2	TNF	IFN-γ	IL-4	IL-6	IL-10	IL-17A
SIN vs REP	0.10	0.07	0.30*	0.33*	0.27	0.22	0.04

* $p < 0.05$.

Discussion

This is the first study to analyse changes in Th1, Th2 and Th17 cytokine production in response to repeated exercise. Data were presented as absolute cytokine production following whole blood stimulation by PMA and ionomycin, and adjusted according to total leucocyte concentration at each corresponding time point. A significant increase in leucocyte concentration was present at each exercise bout, and there was a significant difference between trials at post-A.M.-Ex and pre-P.M.-Ex. Regarding Th1 cytokines, there was no significant difference between SIN and REP trials for IL-2, TNF, or IFN-γ, however there was a trend towards a significant time x trial interaction in IL-2. There was a significant increase from pre-exercise to post-P.M.-Ex in the SIN trial for IL-2 (both absolute and adjusted values), from pre-exercise to post-P.M.-Ex in the SIN trial for TNF (adjusted value), and from pre-exercise to post-P.M.-Ex in the SIN trial for IFN-γ (adjusted value). For Th2 cytokines there was no significant difference between SIN and REP trials for IL-4, IL-6, or IL-10, however there was a trend towards a significant time x trial interaction in IL-4. For IL-4, there was a significant increase from pre-exercise to pre-P.M.-Ex, post-P.M.-Ex, and 1 h, 2 h, and 4 h post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex, post-P.M.-Ex, and 2 h post-P.M.-Ex in the REP trial. There was no significant change from pre-exercise in IL-6 production. For IL-10, there was a significant increase from pre-exercise to 1 h, and 4 h post-P.M.-Ex in the SIN trial, and from pre-exercise to pre-P.M.-Ex in the REP trial. Finally, there was no significant difference between trials, nor a significant change from pre-exercise in IL-17A (Th17), however there was a significant time x trial interaction.

There was a significant increase in the absolute concentrations of IL-2 (Th1), IL-4, and IL-10 (both Th2) to post-exercise in the single trial, whereas in the repeat trial IL-4 and IL-10 significantly increased. While the increase following the single trial appears to be exercise-induced, this is not as clear in the repeat trial. There was no significant difference between SIN and REP trials including during the A.M.-Ex bout. When examining the repeated bouts, it would appear that exercise had no detectable effect on mitogen-stimulated cytokine production, and rather that the significant increase in production of IL-2, IL-4, and IL-10 were driven by normal daily changes (diurnal?) in cellular function. For IL-2

and IL-4 there was a trend towards a significant time x trial interaction. This suggests a difference in the change in cytokine concentration between single and repeat bouts over the period of collection. Potentially, the repeated exercise bout may alter the changes in cytokine IL-2 and IL-4 compared to a single bout of exercise, although this is not as clear cut as an 'carry-over' or 'additive effect' as previously described [177-179, 186-188]. Rather, the pattern of change post-exercise (or pre-P.M.-Ex) may be causing the interaction effect. In particular, from pre- to post-P.M.-Ex there appears to be contrast in the change in cytokine concentration between the two trials; for both IL-2 and IL-4 the single trial is increasing in concentration while the repeat trial is decreasing. It may be possible that the A.M.-Ex drove cytokine production after exercise, only to decrease during the second bout, while the single bout of exercise drove that same increase in the single trial during P.M.-Ex.

As mentioned above, IL-17A did not significantly change at any time point, nor was there a significant difference between SIN and REP trials. However, there was a significant time x trial interaction present. This suggests a difference the change in cytokine concentration between single and repeat bouts over the period of collection. When examining the two trials, the pattern of change appears quite different. It appears that A.M.-Ex increased cytokine production after exercise, only to decrease during the second bout, while the single bout of exercise drove that same increase in the single trial during P.M.-Ex. Potentially, this may be demonstrating the effect of repeated exercise on IL-17A production. IL-17A is primarily released by Th17 cells. The Th17 subset is the first $CD4^+$ subset to be generated allowing rapid response against infection [49]. If repeated exercise does effect the ability for IL-17A to be produced, this may be of concern when considering rapid response to infection. Previous research has demonstrated that IL-17 is induced by IL-6 and activated by IL-23 which may promote neutrophil activation following prolonged endurance exercise [337]. When Spearman's correlation analysis was used to examine the relationship between IL-6 and IL-17A, a significant positive correlation ($r = 0.43$) was observed. When considered in the context of all cytokine associations of the present study, this association would be considered quite weak, as mitogen stimulation is likely to not

demonstrate the same effect as IL-6 would have on IL-17A production with no other stimulant present.

As the assay used was a whole blood analysis, cytokine production was normalised according to leucocyte concentration at each time point. This was to allow comparison of function on a "per cell" scale. This analysis demonstrated a significant increase from pre-exercise in Th1 cytokines; IL-2, TNF, and IFN-γ, and Th2 cytokines; IL-4 and IL-10. It is difficult to determine if this increase in production is due to the exercise intervention, as the time course of changes does not follow that classic cellular redistribution kinetics during the recovery period [1, 197]. Compared to changes in leucocyte concentrations following acute stress or exercise, less is known about cytokine production following exercise, as few studies have examined changes during the post-exercise period. Previously, intracellular cytokines IL-2 (Th1), IFN-γ (Th1), and IL-4 (Th2) produced by $CD4^+$ T cells were used to define T helper cell types and their functional changes. Using this method, a Th1-to-Th2 "shift" was described following exercise and hormone infusion [155, 167, 168, 310]. This 'shift' occurs when IL-2- and/or IFN-γ-producing T helper cells (Th1) decrease in cell concentration, while IL-4-producing T helper cells (Th2) remain unchanged. It is clear the present study did no show such changes. Compared to intracellular analysis of cytokine production, whole blood mitogen-stimulation analysis is a much more complex display of the cytokine network. Cytokines are not solely secreted from T helper cells, but include cytotoxic T cells, natural killer cells, dendritic cells, macrophages and even skeletal muscle [311, 312]. This suggests there is a much more complex array of interactions that are occurring following exercise-induced stress. When examining IFN-γ and TNF there was a significant increase in adjusted concentration immediately post-exercise in the single trial (but not the repeat trial). As there was no significant increase in absolute IFN-γ and TNF concentrations at this time point, it would suggest that this was driven by a change in the relationship between leucocyte concentration and cytokine concentration. This occurred immediately after the single exercise bout when mean concentration was at its peak (although not significant), and cytokine production per cell was also at its peak. This may suggest exercise increased immune cells ability to produce cytokines, unfortunately this was not a reproducible

change, it did not occur following repeat bouts of exercise. Previously, following a 6-h endurance run, LPS-stimulated production of TNF decreased post-exercise [338]. This was not seen in the present study, which may be due to the mode of stimulation, or potentially the duration of the exercise bout.

Whole blood mitogen-stimulation provides a challenge to the cellular immune system. This is because a stressor is applied (PMA and ionomycin) to drive cytokine production after an initial stressor is applied (exercise), which is also reported to alter cytokine production [96, 339]. The resultant changes in cytokine concentrations between sampling points are then used to determine the influence of exercise on this production. In the present study, exercise would appear to have minimal effect on leucocyte ability to produce cytokines following mitogenic stimulation. Although, previous research has largely used PHA-stimulation of PBMCs to analyse the above effect, conflicting results still remain. In one study, IFN-γ concentration significantly increased from pre-exercise to 24 h following moderate, but not exhaustive exercise, whereas no significant change was reported in IL-2 concentration [254]. Whereas, no significant change was apparent in IFN-γ or IL-2 production following PHA-stimulation of PBMCs after cycling for 60 min at 75 % of $\dot{V}O_2$peak [255]. Conversely, following cycling for both, 60 min at 60 % of $\dot{V}O_2$peak, and a 19 min exhaustive effort, PHA-stimulation of PBMCs resulted in a significant decrease in IL-2 production [250, 256]. These results did use a different stimulant and highlights the variable nature of previous results examining this phenomenon. When associations between individual cytokines at each time point were examined using Pearson's correlation analysis, there was a significant correlation amongst all cytokines, ranging from p=0.93 to p=0.34. This may suggest that while results were quite variable over time, stimulation at each time point appeared to drive a similar response in all cytokines, suggesting PMA and ionomycin are quite suitable as stimuli for Th1/Th2/Th17 cytokines, although perhaps not when examining the effect of exercise on cytokine production.

There is certainly a trade-off between the quantity and the specificity of data when examining cytokine production following mitogen stimulation. One argument is that the cytokines are not just produced by T helper cells, but include cytotoxic T cells, natural

killer cells, dendritic cells, macrophages and even skeletal muscle [311, 312]. Hence, to examine a single cell type and production of one or two selected cytokines may not present a normal physiological response. Conversely, whole blood stimulation over multiple time points appears to be a hugely complex array of changes, often making it difficult to highlight the significance of results. The present study suggests prior exercise didn't have an effect on cytokine production except for perhaps IL-17A, which may be important regarding rapid response to infection.

6. STUDY IIIB – REPEATED PROLONGED HEAVY EXERCISE AND ITS EFFECT ON CELLULAR REDISTRIBUTION

Abstract

The purpose of this study was to investigate the effects of prior heavy-intensity exercise on circulating dendritic cell subpopulations, γδ T cell subpopulations and memory phenotypes, and subsequent changes in these cells following a second bout of exercise. Ten well-trained endurance cyclists (mean ± SD: age 23.2 ± 3.2 years; height 1.82 ± 0.06 m; body mass 74.8 ± 6.2 kg; peak oxygen uptake 59.0 ± 4.3 $mL.kg^{-1}.min^{-1}$) performed two exercise trials on different occasions. On the first occasion, they cycled in the morning and afternoon for 1.5 h at 90% of their second ventilatory threshold, with 4 h between the two bouts. On the second occasion, they cycled for 1.5 h at the same intensity, but only in the afternoon. Blood samples were collected throughout the recovery period post-exercise and flow cytometry was used to examine changes in cell numbers and cell-surface receptor expression. Significant exercise-induced increases were observed in total lymphocytes, neutrophils, total γδ T cells, δ1 and δ2 subsets, total dendritic cells, and the plasmacytoid dendritic cell subset concentrations. Furthermore, among the γδ T cells, those with high expression of CD11a, CD62L, and CD94 were preferentially mobilised. However, no significant differences were observed between the single and repeated exercise trials for any variable, except total lymphocytes, neutrophils, and plasmacytoid DCs. Indeed, many immune markers (particularly total lymphocytes and related subsets) featured near identical recovery patterns between the single and repeated exercise bouts. In contrast to previous findings, prior exercise may have less effect on lymphocyte redistribution following a subsequent bout of prolonged heavy-intensity exercise than previously suggested. Energy intake between exercise trials may be an important determinant of these responses.

Introduction

Regular heavy-intensity endurance exercise is associated with transient changes in circulating leucocyte concentrations, immune cell function, and potentially, an increased risk of upper respiratory illnesses (URIs) [1-5]. Previous research has demonstrated that acute stress (such as regular exercise) induces positive effects on the immune system, whereas chronic stress is considered harmful [102, 108]. In their training, endurance athletes regularly challenge the balance between a healthy immune system and training-induced immunosuppression [34, 36, 334]. Notably, the increased incidence of symptoms associated with URIs in elite athletes regularly occurs during periods of heavy training and competition [3-5, 24, 32, 335], which are associated with regular and more severe stressors, and decreased recovery time. Repeated exercise, without sufficient recovery, may cause suppression in immune function, which may in turn increase susceptibility to URIs [76, 108, 336].

Various studies have examined the influence of successive bouts of exercise on immune function [156, 177-188], with several of these studies highlighting a 'carry-over' or 'additive' effect of on lymphocyte and neutrophil concentration changes [177-179, 186-188]. Potentially, these carry-over effects may lead to chronic suppression of immune function [76]. Repeated exercise appears to induce a more pronounced increase in catecholamine and cortisol concentrations compared to a single bout, resulting in a more severe ingress of lymphocytes and neutrophils into peripheral circulation [177, 179]. It is largely unknown how prior intense exercise alters the cellular immune system during and following a subsequent exercise bout. To date, only neutrophils and lymphocytes (including $CD4^+$, $CD8^+$ and $CD56^+$ cells) have been examined within this context [177-179, 186-188]. No research has investigated the effects of repeated bouts of exercise on a number of lymphocyte subsets or dendritic cells.

γδ T cells have been labelled 'the bridge between innate and adaptive immunity'; they play an important role in early immune responses to a variety of pathogens [78]. Displaying a wide array of functions, γδ T cells are capable of MHC-independent activation, T cell receptor-dependent recognition of conserved antigens, and professional antigen

presentation, while possessing protective memory and reactive memory capabilities [78, 79]. αβ T cells are involved in adaptive immunity, and are abundant in the peripheral circulation. Previous research has extensively examined these cells in response to stress and exercise [1, 157, 179, 340]. Conversely, γδ T cells constitute only 1-5% of T lymphocytes in the blood stream in humans and mice [80]. These cells are, however, found in abundance in the epithelia of skin, genital, intestinal and respiratory tract [81-83]. These areas are regularly expose to an array pathogens; consequently, γδ T cells play an important role in early innate recognition and antigen presentation in response to a variety of invading agents. Previous research has demonstrated that acute exercise and psychological stress stimulates ingress of γδ T cells with high cytotoxic capabilities, tissue homing potential, and the capacity for rapid, innate-like target recognition [104, 160].

Dendritic cells (DCs) function as professional antigen-presenting cells (APC)s in the blood, and appear to have immature/precursor characteristics, because they undergo a maturation process after exiting the circulation [63]. These cells play a crucial role in initiating and regulating both cellular and humoral immune responses. Previous research has revealed conflicting changes in redistribution of these cell types, following a marathon run. In particular, mDCs in circulation increased significantly, whereas pDCs decreased significantly below pre-exercise values [164]. Following a 1-hour ice-hockey training session, circulating mDC and pDC concentrations both increased significantly compared with rest [165]. No studies to our knowledge have examined changes in DCs under conditions where exercise intensity and duration are controlled, nor in response to repeated exercise bouts.

Exercise-induced stress alters the distribution and function of immune cells for hours after the end of exercise. Questions remain, however, regarding the effect of repeated exercise on immune cell redistribution and function. Few studies have examined the immune response to successive exercise bouts. Specifically, a number of cell types including γδ T cells and dendritic cells have not been examined at all under these conditions, but are known to respond to acute stress, whether exercise, psychological stress, surgery, or β-agonist infusion [104, 105, 164]. Previous research has highlighted a greater cellular

ingress of $CD4^+$ and $CD8^+$ T cells in response to a second bout of exercise [179]. This study aimed to examine the influence of prior heavy endurance exercise on the subsequent cellular immune system changes during a second exercise bout. Specifically, changes in the numbers of circulating leucocytes, γδ T cells and dendritic cells were measured during and after exercise that was performed 4 hours after an initial exercise bout. It was hypothesized that due to a repeated, prolonged exercise-induced stress, the cellular ingress would be greater in the subsequent bout of exercise.

Methods

Subjects

Ten highly-trained male cyclists volunteered to participate in the present study. Physical characteristics of the cyclists are presented in Table 6.1. Subjects were excluded if they exhibited any cardiovascular, metabolic, neurological, or autoimmune disorders. The cyclists provided written informed consent for participation in the study, which was approved by the Bond University Human Research Ethics Committee.

Table 6.1. Physical characteristics, aerobic capacity, and threshold performance data

Subject Characteristics (n=10)	Mean ± SD
Age (years)	23.2 ± 3.2
Height (m)	1.82 ± 0.06
Body mass (kg)	74.8 ± 6.2
BMI ($kg.m^{-2}$)	22.5 ± 1.6
Absolute $\dot{V}O_2$peak ($L.min^{-1}$)	4.4 ± 0.3
Relative $\dot{V}O_2$peak ($mL.kg^{-1}.min^{-1}$)	59.0 ± 4.3
Peak Power (W)	411 ± 28
Peak Power-to-weight (W/kg)	5.5 ± 0.5
VT_1 Power (W)	287 ± 23
VT_1 Power-to-weight (W/kg)	3.8 ± 0.3
VT_2 Power (W)	342 ± 30
VT_2 Power-to-weight (W/kg)	4.6 ± 0.4
Training (km/week	316 ± 75

Data are mean ± standard deviation. BMI: body mass index. $\dot{V}O_2$peak: peak oxygen uptake. VT_1: first ventilatory threshold. VT_2: second ventilatory threshold. Training: volume in kilometres performed during training per week.

Experimental Design

Each cyclist visited the laboratory on four occasions; for a screening/familiarization session, a maximal exercise test, and two experimental exercise sessions. The experimental exercise sessions (Figure 6.1) involved each cyclist completing both a morning (A.M.-Ex)

and afternoon (P.M.-Ex) bout of exercise (Repeat Trial - REP), and on a separate occasion, only an afternoon bout of exercise (Single Trial - SIN). The two experimental trials were separated by a minimum of seven days, during which time the cyclists were permitted to train as normal. Participants completed the SIN and REP trial in a counter-balanced order. Throughout the testing period, cyclists were required to report any upper respiratory or gastrointestinal symptoms. All cyclists refrained from exercise for 48 h prior to both the maximal exercise test and the experimental trials. Alcohol or caffeine consumption was not permitted during the exercise and post-exercise sample period.

Peak oxygen consumption ($\dot{V}O_2$peak) was determined during a graded exercise test in which the participants cycled at their preferred cadence within the range of 70–100 rev.min^{-1}. The test was performed on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The cyclists commenced the graded exercise test at 90 W for 4 min, after which the work rate increased by 30 W/min until volitional fatigue and/or when the cyclists could not maintain their cadence above 70 rev.min^{-1}, despite strong verbal encouragement. The criteria used to determine maximal effort at the end of the test were: (i) a plateau in $\dot{V}O_2$ (defined as an increase in $\dot{V}O_2$ values of less than 2 mL.kg^{-1}.min^{-1} between two consecutive 1-min periods in the final stages of the test), (ii) a maximal respiratory exchange ratio $\geq$ 1.1 and/or (iii) a maximal heart rate within 5% of their individual age-predicted maximum (220-age) [304]. Each cyclist's first (VT_1) and second (VT_2) ventilatory threshold was determined from the gas-exchange data collected during incremental exercise (ParvoMedics' TrueOne® 2400, Utah, USA), using the ventilatory equivalents methods [267].

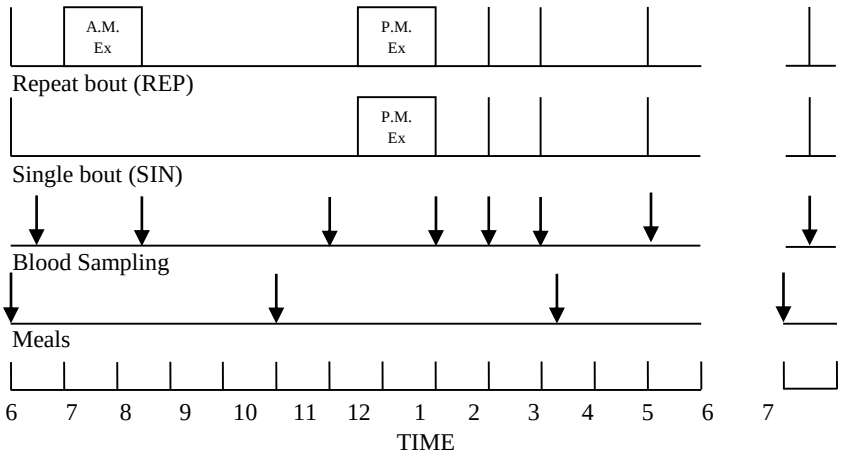

Figure 6.1. Exercise, blood collection, and meal schedule for the Single and Repeat bout data collection protocols. Boxes represent exercise bouts. Arrows represent times of blood collection and scheduled meals.

On arrival for the SIN or REP trials, the cyclists consumed a standardised breakfast (2,000 kJ) of cereal, milk, and juice. Pre-exercise blood samples were collected from the cyclists following breakfast. On the day of the REP trial, the cyclists exercised for 1.5 h at 90% VT_2 on a stationary cycle ergometer (Monark Ergomedic 828 E, Sweden) beginning at 7:00 a.m. (A.M.-Ex) and again at 12:30 p.m. (P.M.-Ex). For the SIN trial, the cyclists also exercised for 1.5 h at 90% VT_2, but only once, beginning at 12:30 p.m. (P.M.-Ex). Heart rate was monitored continuously throughout exercise. Pulmonary gas exchange was sampled for 4 min at 20-min intervals during the exercise bouts to ensure that the cyclists were consistently exercising at the work rate associated with 90% VT_2, and that no drift in $\dot{V}O_2$ occurred. The cyclists ingested water *ad libitum* during exercise. Immediately after exercise, a venous blood sample was collected. In total, eight blood samples were collected at 6.30 a.m. (rest), 8.30 a.m. (post A.M.-Ex), 12.00 p.m. (pre P.M.-Ex), 2.00 p.m. (post P.M.-Ex), 3.00 p.m. (1-h post P.M.-Ex), 4.00 p.m. (2-h post P.M.-Ex), 6.00 p.m. (4-h post P.M.-Ex) and 6.30 a.m. (24-h post rest) on the subsequent day. A post-exercise snack was provided at 8.45 a.m. and 2.15 p.m. consisting of breakfast cereal, milk and honey (1,500 kJ), while larger meals were provided to the cyclists at 11.30 a.m. and 4.15 p.m. consisting of sandwiches and a milkshake (4,500 kJ).

Flow cytometry

Venous blood was sampled from an antecubital vein and collected into blood collection tubes containing K_3EDTA (BD Vacutainer, New Jersey, USA). Samples were analysed to determine the full blood cell count (Coulter HmX Hematology Analyzer, Beckman Coulter). Percent change in blood, cell and plasma volumes were calculated for each sample [291], and subsequently all cell concentrations were adjusted for shifts in blood volume. Cells were identified by immunofluorescent antibody staining of whole blood using four-colour flow cytometry (FACS-Calibur, BD Biosciences).

Lymphocyte subsets

Total γδ T cells, δ1 and δ2 subsets, memory phenotypes, and surface molecules were determined using the following antibodies; CD3 PerCP (BD Biosciences), Vδ-1 TCR FITC (Thermo Scientific), γδ TCR APC, Vδ-2 TCR APC, CD27 FITC, CD27 PE, CD45RA APC, CD11a FITC, CD62L PE, CD94 FITC (BD Pharmingen) (Anane et al., 2010). Briefly, 100 µL whole blood were incubated with appropriate concentrations of antibody (as determined by titration) for 30 min in the dark at room temperature. Erythrocytes were subsequently lysed using FACS lysing solution (BD Bioscience). Following lysis, samples were centrifuged (350 × *g*, 5 min, at room temperature). Samples were then washed and fixed in 1% paraformaldehyde. Fixed preparations were analysed immediately, collecting a minimum of 100,000 events from each sample. Appropriate, matched isotype controls were used to set negative staining criteria. The FACS-Calibur flow cytometer (BD Biosciences) was calibrated weekly using Calibrite beads (BD Biosciences). Total lymphocytes were gated using the forward versus side scatter plot to identify the target population. γδ T cells were then gated as the $CD3^+$ γδ TCR^+ population, δ1 T cell were gated as $CD3^+V\delta1^+$ and δ2 T cells were gated as $CD3^+$ $V\delta2^+$. Lymphocyte subset concentrations were quantified using the percentage values obtained from each lymphocyte subset gate. The total lymphocyte concentration was obtained from the full blood count.

Dendritic cell phenotypes

Total dendritic cells (tDCs), dendritic cell phenotypes (pDCs, mDCs, $CD14^{-/low}CD16^{+}$ DCs), and total monocytes were identified by the combination of HLA-DR PerCP, lineage cocktail-2 FITC (CD3, CD14, CD19, CD20, CD56), CD123 PE (BD Bioscience), CD33 APC, CD11c PE and CD16 APC (BD Pharmingen) antibodies. tDCs were identified based on the expression of CD33 and HLA-DR, and the absence of the lineage cocktail-2 antigens with intermediate forward and side scatter characteristics located between lymphocytes and monocytes. Dendritic cells were further defined into three subpopulations: (1) mDCs , which included a subset of $CD123^{dim}/CD33^{high}/CD16^{-}$ cells, with strong expression of HLA-DR; (2) a subset of $CD123^{inter}/CD33^{inter}/CD16^{+}$ cells– $CD14^{-/low}CD16^{+}$ DCs, and (3) pDCs, which included $CD123^{high}/CD33^{-/dim}/CD16^{-}$ cells, both with intermediate expression of HLA-DR [289]. Cell concentrations were quantified using percentage values obtained from each lymphocyte and monocyte subset gate. The total monocyte concentration was obtained from the full blood count.

Statistics

All data are presented as mean ± standard deviation. All statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, USA). Performance measures (heart rate, cadence, power output) across the three exercise trials were compared using one-way ANOVA with repeated measures. All immunological data were analysed using a 2 (single and repeated exercise bouts) x 8 (times of measurement) repeated measures ANOVA. Data were assessed for the assumptions of normality, homogeneity of variance and sphericity. If the assumption of sphericity was violated, the degrees of freedom were adjusted using the Greenhouse-Geisser correction. The time × trial interaction effect is reported. Where significant effects between trials were found ($p<0.05$), separate paired t-tests with Bonferroni corrections for multiple comparisons were performed between each time point across the two trials (SIN versus REP trial). If significant time effects were found ($p<0.05$), separate paired t-tests with Bonferroni corrections for multiple comparisons were performed to compare the pre-exercise resting sample to each subsequent time point.

Results

Physiological variables during exercise

Power output, heart rate and cycling cadence did not differ between exercise trials (Table 6.2).

Table 6.2. Physiological variables during exercise

	SIN P.M.-Ex	REP A.M.-Ex	REP P.M.-Ex	P value
Mean HR (beats.min^{-1})	151 ± 11	150 ± 11	150 ± 10	0.359
Mean Cadence (revolutions.min^{-1})	87.9 ± 7.7	87.3 ± 7.8	87.7 ± 7.9	0.089
Mean Power Output (W)	297.0 ± 28.5	297.4 ± 27.4	294.5 ± 27.0	0.143

Data are mean ± standard deviation. HR: heart rate.

Leucocyte concentrations

Difference between trials. Before the start of P.M.-Ex (REP), changes in cell concentrations due to A.M.-Ex (REP) had returned to resting values in the lymphocyte and monocyte subpopulations. Immediately post-A.M.-Ex lymphocyte and neutrophil concentrations were significantly higher in the REP trial, compared to the SIN trial. The neutrophil concentration of the REP trial continued to be significantly above the SIN trial at pre-P.M.-Ex.

Effect of exercise. When trials were examined individually, all exercise bouts resulted in an increase in lymphocyte, neutrophil and monocyte concentrations (all $p<0.001$, Figure 6.2).

γδ T cells

Difference between trials. When comparing between bouts, there was a significant time x trial interaction for total γδ T cells ($p<0.05$) and the δ1 subset ($p<0.05$). Further analysis demonstrated a trend towards a main effect between trials (δ1; $p=0.059$, γδ T cells; $p=0.052$).

Effect of exercise. A significant time effect was present for total γδ T cells, δ1, and δ2 subsets. A significant increase was evident in both the SIN and REP trials for total γδ T cell concentration, and δ1 and δ2 subset concentrations (all $p<0.05$, Figure 6.3).

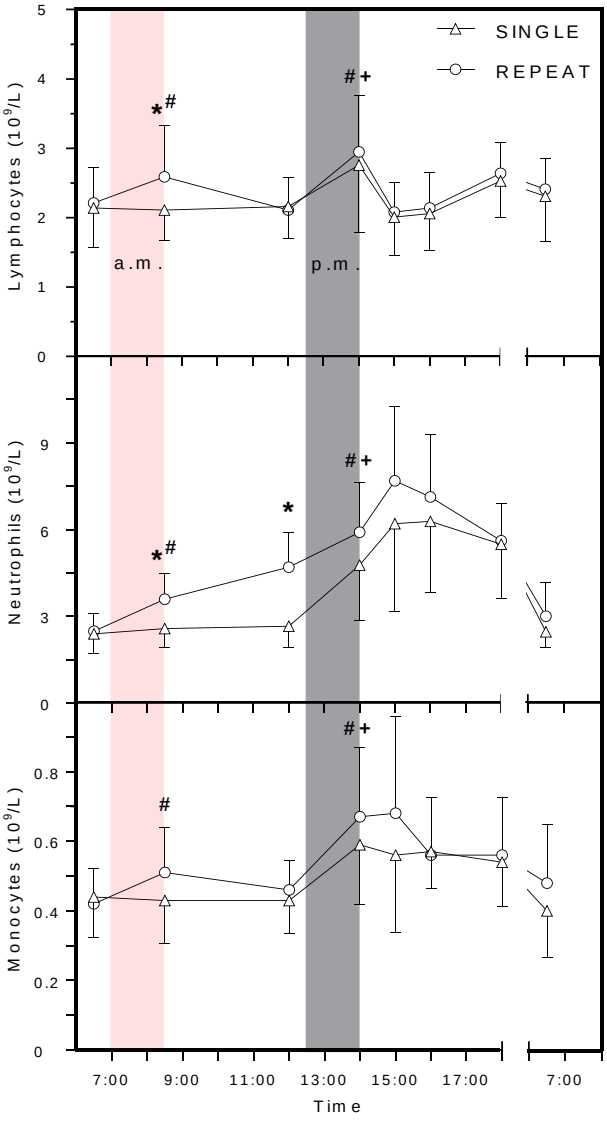

Figure 6.2. Total lymphocyte, neutrophil, and monocyte concentrations before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. Lymphocytes: 0.266; <0.001; 0.116. Neutrophils: 0.197; <0.001; 0.039. Monocytes: 0.125; <0.001; 0.115. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, signifies a significant difference between trials at time point. #, signifies a significant difference from pre- to immediately post-exercise for the REP trial. +, signifies a significant difference from pre- to immediately post-exercise for the SIN trial.

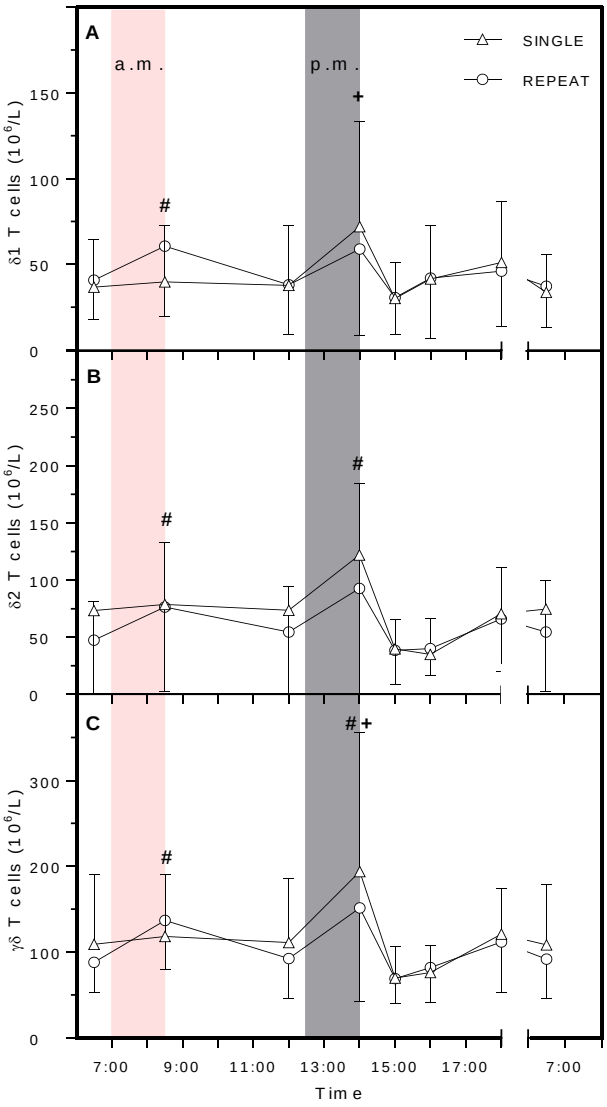

Figure 6.3. Total γδ T cells (A), δ1 (B) and δ2 (C) subset concentrations before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. δ1: 0.012; 0.027; 0.059. δ2: 0.132; 0.016; 0.125. γδ T cells: 0.031; 0.002; 0.052. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. #, signifies a significant difference from pre- to immediately post-exercise in the REP trial. +, signifies a significant difference from pre- to immediately post-exercise for the SIN trial.

Memory phenotypes

Difference between trials. No significant difference was observed between the two trials, both during exercise and recovery.

Effect of exercise. All memory phenotypes did not exhibit any significant time effect as a result of the exercise bouts (Figure 6.4). There was a trend towards significance in EMRA (p=0.082) and CM (p=0.063) phenotypes.

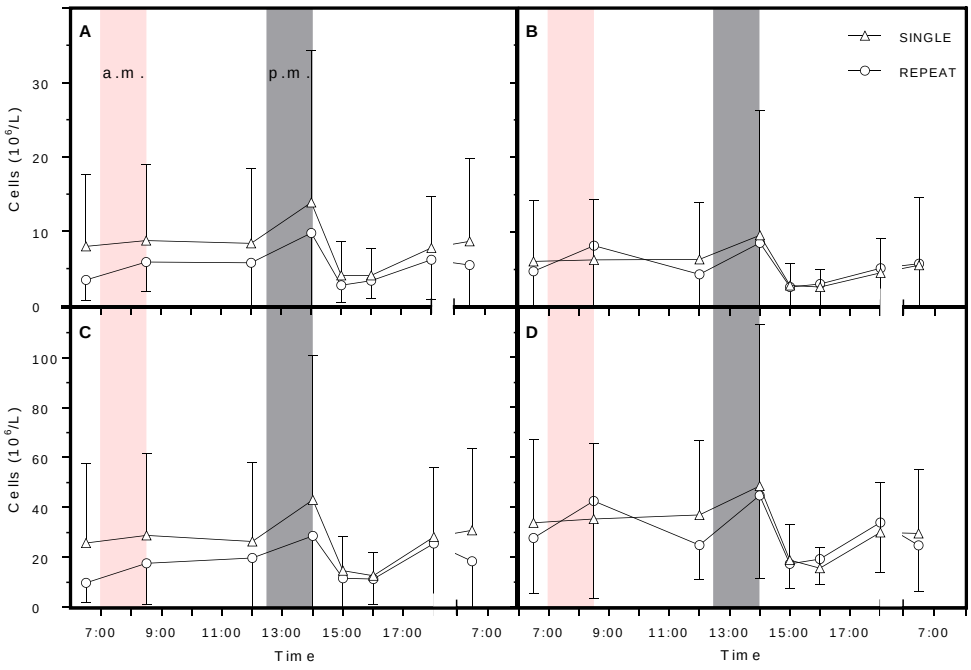

Figure 6.4. δ2 CD45RA$^+$ effector memory (A), effector memory (B), naïve (C), and central memory (D) phenotype concentrations before and after a bout of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. EMRA: 0.297; 0.082; 0.142. EM: 0.306; 0.127; 0.262. NA: 0.248; 0.109; 0.129. CM: 0.192; 0.063; 0.164. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial.

Receptor expression

Difference between trials. When comparing between bouts, the pattern of change did not differ between trials, both during exercise and subsequent recovery period. There was a trend towards a significant time x trial interaction in $CD94^-$ (p=0.063).

Effect of exercise. A significant exercise-induced increase was evident immediately following P.M.-Ex in $CD11a^{hi}$, $CD62L^{hi}$, and $CD94^+$ γδ T cell concentrations (all p<0.05, Figure 6.5, 6.6, 6.7).

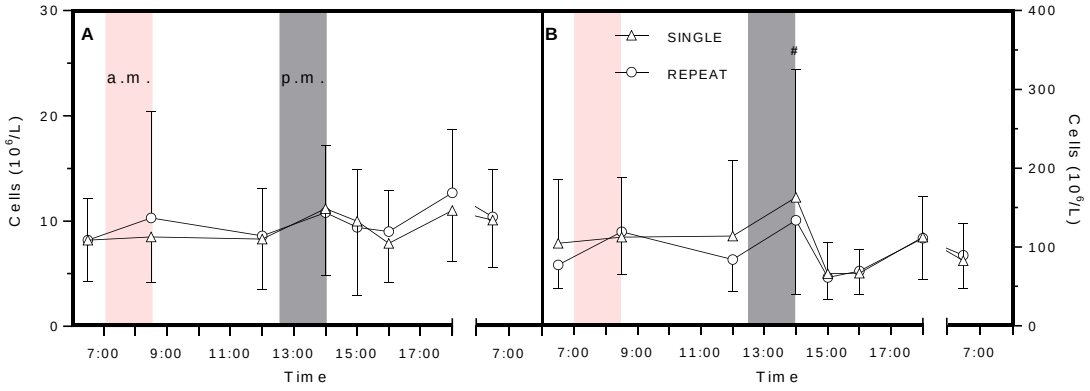

Figure 6.5. Total γδ T cell $CD11a^{low}$ (A) and $CD11a^{high}$ (B) expression before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. $CD11a^{low}$: 0.137; 0.765; 0.347. $CD11a^{high}$: 0.149; 0.023; 0.107. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. #, signifies a significant difference from pre- to immediately post-exercise in the REP trial.

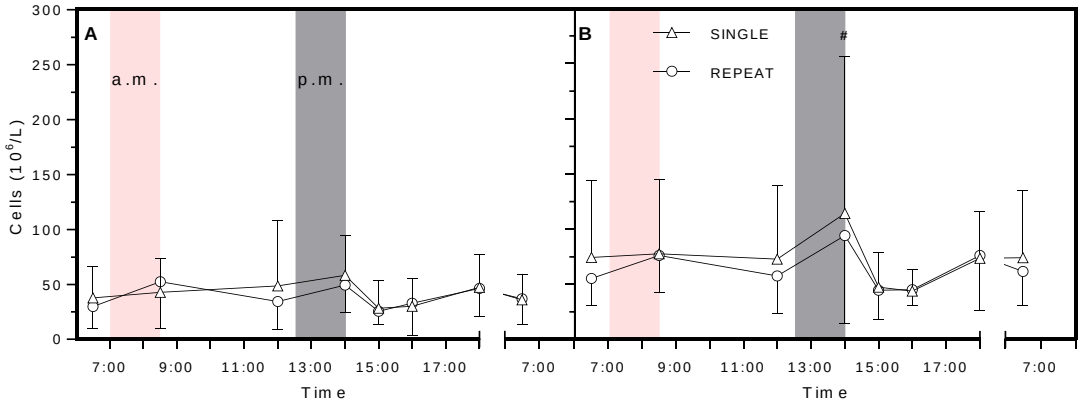

Figure 6.6. Total γδ T cell CD62L^{low} (A) and CD62L^{high} (B) expression before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. CD62L^{low}: 0.221; 0.181; 0.350. CD62L^{high}: 0.191; 0.003; 0.175. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. #, signifies a significant difference from pre- to immediately post-exercise in the REP trial.

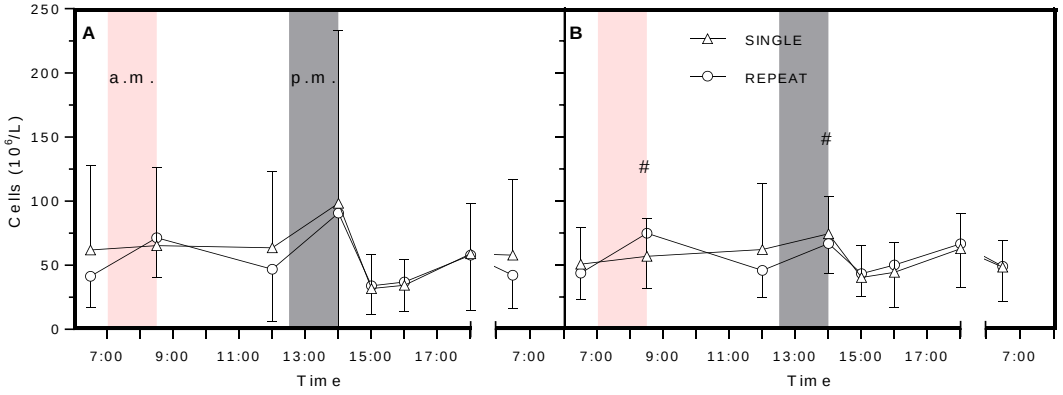

Figure 6.7. Total γδ T cell CD94^{-} (A) and CD94^{+} (B) expression before and after single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. Time x trial interaction: Main effect of time: Main effect of trial. CD94^{-}: 0.063; 0.086; 0.060. CD94^{+}: 0.138; 0.050; 0.136. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p

value represents the main effect of trial. #, signifies a significant difference from pre- to immediately post-exercise in the REP trial.

Dendritic cell phenotypes

Difference between trials. A significant trial x time interaction was evident in $CD16^+$ DCs and total DCs. pDCs were significantly higher in the REP trial at post-A.M.-Ex compared to the SIN trial.

Effect of exercise. A significant exercise-induced increase in concentration was evident in tDCs, and pDCs following P.M.-Ex ($p<0.05$, Figure 6.8).

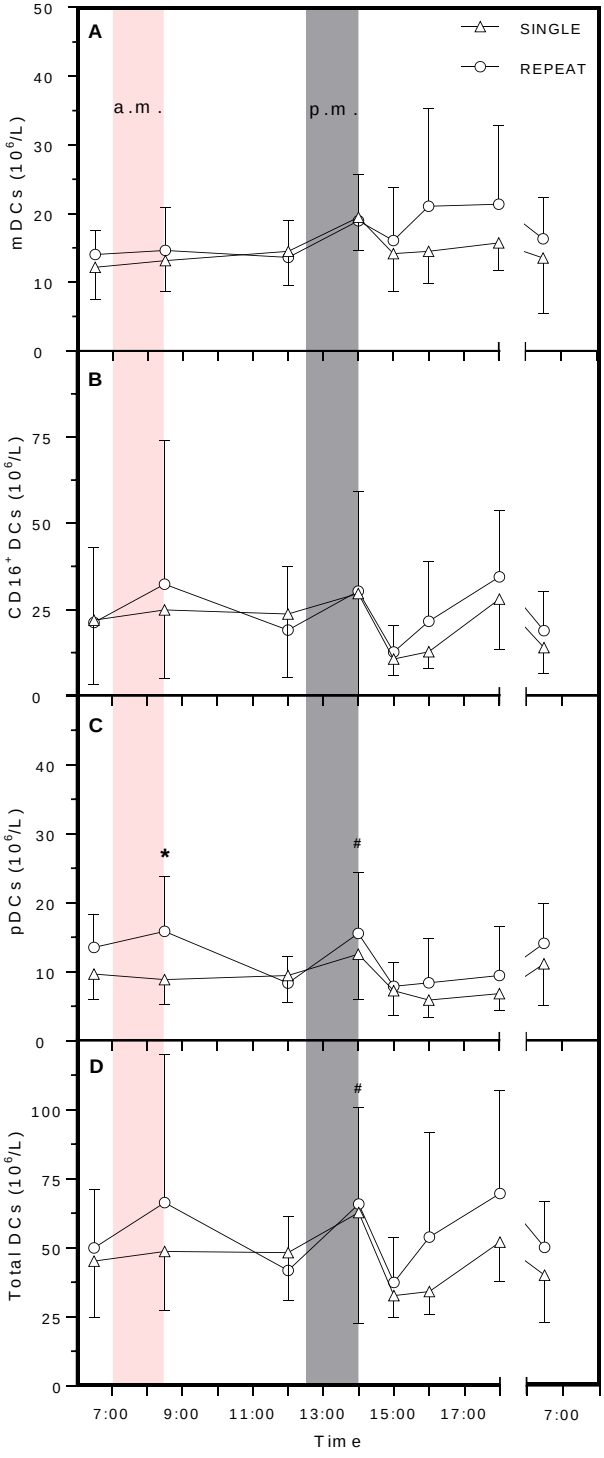

Figure 6.8. Dendritic phenotype subsets; mDC (A), $CD16^+$ (B), and pDC (C) and total dendritic cell (D) concentrations single and repeat bouts of prolonged heavy-intensity exercise. Data represent mean ± SD. tDC: total dendritic cells. mDC: myeloid dendritic cells. pDC: lymphoplasmacytoid dendritic cells. $CD14^{-/low}CD16^+$ DC: monocyte-related dendritic cells. Time x trial interaction: Main effect of time: Main effect of trial. mDC: 0.523; 0.243; 0.220. $CD16^+$ DC: 0.004; 0.275; 0.224. pDC: 0.116; 0.013; 0.001. Total DC: 0.040;

0.036; 0.117. The first p value represents the trial (single versus repeat) x time interaction; the second p value represents the main effect of time; the third p value represents the main effect of trial. *, signifies a significant difference between trials at time point. #, signifies a significant difference from pre- to immediately post-exercise in the REP trial.

Discussion

The main finding of this study was that prior heavy exercise did not alter the time course of redistribution of circulating immune cells following a second bout of prolonged heavy-intensity exercise performed on the same day. While neutrophil concentration remained elevated immediately prior to the start of the second exercise bout, lymphocyte concentration, total γδ T cells, δ1 and δ2 subsets, memory phenotypes, and surface receptors, and dendritic cells did not significantly differ between the SIN and REP trials following P.M.-Ex, however there was a significant time x trial interaction in total γδ T cells, δ1 subset, $CD16^+$ DCs and total DCs. Additionally, despite the increased neutrophil concentration before the second cycling bout (P.M.-Ex), prior heavy exercise did not influence the neutrophil response during the exercise period, nor the time course of neutrophil recovery following exercise. Indeed, many of the subsets (particularly total lymphocyte concentration and its related subsets) featured near identical recovery patterns following P.M.-Ex between both trials. These results are important, in contrast to previous findings, prior exercise may have less effect on lymphocyte cell redistribution following a subsequent bout of prolonged heavy-intensity exercise than previously suggested.

An increase in neutrophil concentration immediately before the second exercise bout in the REP trial in the present study is consistent with previous findings [178, 179, 186-188]. The elevated neutrophil response resulted in a more pronounced change in neutrophil concentration following the subsequent bout of exercise performed on the same day. A collection of publications from Ronsen et al monitored nine endurance trained athletes who completed one or two bouts of cycling for 75 min at ~75% $\dot{V}O_2$peak separated by a 3 h or 6 h rest period [177-179, 188]. Following the second bout of endurance exercise, comparing single and repeated protocols, there was a significant increase in neutrophil, lymphocyte, $CD4^+$, $CD8^+$, and $CD56^+$ cell concentrations, and hormones; adrenaline, noradrenaline, and cortisol [177, 179]. Similar results were reported following 1 h of cycling sub-divided into three segments each of 20 min duration (5 min at 50% $\dot{V}O_2$peak and 15 min at 70% $\dot{V}O_2$peak) on two occasions separated by 4 h. Both neutrophil and lymphocyte concentrations were significantly higher after two bouts of exercise than after the single bout [187]. Another study analysed the immune response to two 2 h bouts of cycling at

60% of $\dot{V}O_2$max, separated by 3 h. Similar results were found in leucocyte concentrations and stress hormones, with a decrease observed in neutrophil function [186]. This redistribution of neutrophils is likely the result of the combined effects of stress hormones, including growth hormone, epinephrine, norepinephrine and cortisol [115, 116, 125, 137, 341, 342]. In contrast to previous findings [179], no difference was observed between trials in lymphocyte concentration following P.M.-Ex. Previous research has observed a greater lymphocytosis during exercise that was performed subsequent to an initial bout. These previous studies utilised exercise protocols of shorter duration and comparable intensity to the present study [178, 179, 187]. In contrast, the present findings are consistent with a study that reported no difference in lymphocyte concentration between repeated exercise bouts that employed 2 h efforts at 60% of $\dot{V}O_2$max [186]. A possible explanation for the inconsistency between these studies may be due to food intake between exercise bouts. Both, McFarlin et al. (2002), and Ronsen et al. (2001a and 2001b), used protocols where muscle glycogen stores were not fully replenished between bouts. Estimated energy expenditure exceeded energy in take by ~1,500 kJ in these two studies [177, 179, 187]. Total energy expenditure for each exercise bout was estimated using reported $\dot{V}O_2$ values during exercise, multiplied by an approximate value of 21 $kJ.L^{-1}.min^{-1}$, multiplied by total minutes of exercise [131]. This value was then compared to the reported energy intake during the study period. Results of this were calculated as ~3,580 kJ expenditure versus 2,125 kJ intake, and ~5,692 kJ expenditure versus 4,184 kJ intake, respectively [177, 179, 187]. One explanation for a greater stress hormone response (particularly epinephrine), and resultant increase in circulating lymphocyte concentration, following the second exercise bout, has been explained by decreased blood glucose levels [177]. In the present study, we attempted to match energy expenditure and intake (~6141 kJ expenditure versus 6000 kJ intake), which we expected would help to replenish muscle glycogen as much as possible before P.M.-Ex. In the study by Li and Gleeson (2004), participants were not fed for the entirety of the sampling period between the two 2-h exercise bouts. This exercise duration coupled with the lack of meals, would be expected to drive a greater rise in lymphocyte concentration following the second bout. This is due to the combined effects of increased exercise duration [126], and low blood glucose concentration [196], on circulating stress hormone concentrations, and the resultant effect on cellular ingress. However, the subjects

were only able to complete ~75 min of the proposed 120 min afternoon exercise protocol [186]. Previous research has demonstrated that exercise duration influences the severity of lymphocytosis [156, 157]. Potentially, in the study by Li and Gleeson (2004), the effect of carbohydrate depletion on stress hormone release was offset by the significantly shorter exercise duration, to cause a similar post-exercise lymphocytosis between single and repeat trials. Together, these results may suggest that matching adequate energy intake before or during exercise to total work performed is imperative to minimise disturbances in leucocyte distribution after exercise. Furthermore, total lymphocyte concentration and its subsets featured very similar recovery patterns post-P.M.-Ex, between SIN and REP trials. This may suggest regardless of prior exercise-induced stress, the leucocyte redistribution follows a distinctly repeatable concentration change in the circulation.

A significant increase was evident immediately following all exercise bouts in δ1 and δ2 subset, and total γδ T cells in the peripheral circulation. However, there were no significant differences in δ1 and δ2 subset, and total γδ T cell concentrations at any time point between SIN and REP trials in the present study. There was however, a significant time x trial interaction for total γδ T cells and the δ1 subset, suggesting a difference in cell concentrations between single and repeat bouts over the period of collection, although this is likely due to the difference in response following A.M.-Ex. Similar to the present study, previous research has demonstrated a significant increase in total γδ T cell concentration [104, 161], and both δ1 and δ2 subsets [163], following short duration exercise (30 min), and in response to psychological stress and β-agonist infusion [104]. These cells are regarded as being highly stress responsive. Ingress into peripheral circulation is driven primarily through β-adrenergic mechanisms causing a release of cells from leucocyte "marginal pools" located within the respiratory circulation, spleen and liver [111, 162, 343]. When the δ2 subset was differentiated into memory phenotypes, no significant difference was present between the two trials at any time point. There was a trend towards a significant time effect for CM (p=0.063) and EMRA (p=0.082). Compared to the results of Study II, this may be a repeatable effect, particularly for CM δ2 γδ T cells. Although there was no immediate post-exercise increase in Study II, both studies featured a decrease (egress) post-exercise in the central memory subset. When analysing expression of CD11a,

CD62L, and CD94 on total γδ T cells, no significant difference at any time point was present between the two trials, however exercise did result in a significant increase in $CD11a^{hi}$ and $CD62L^{hi}$ immediately post-exercise. The response of CD11a reflects similar results following acute psychological stress [163], and has been observed previously in NK and $CD8^+$ T cells [162, 344, 345]. The surface receptor CD11a is involved in cellular adhesion, is typically up-regulated on endothelial cells in proximity of inflamed tissues [92], and is highly sensitive to catecholamine secretion [162]. Immediately post-exercise, a significant increase in γδ T cells expressing high levels of CD62L occurred. CD62L expression reflects lymph node-migratory potential [86]. These exercise-induced results are similar to the response following acute psychological stress [163]. This psychological stress-induced increase in γδ T cells expressing high levels of CD62L was in contrast with findings of CD62L expression on NK and $CD8^+$ T cells following acute stress [318, 344, 345]. However, with the support of the present study, the increase in $CD62L^{hi}$ γδ T cell concentration appears to be a consistent phenomenon. There was a significant increase post-exercise in γδ T cells expressing $CD94^+$, while no significant change was evident for those cells with no expression of CD94 ($CD94^-$). CD94 is involved in innate recognition of aberrant MHC expression on infected and cancerous cells. These results partially resemble previous observations, where psychological stress did drive ingress in both subsets, with a more aggressive mobilisation evident for the $CD94^+$ population [163]. There was a trend towards an exercise-induced time effect of $CD94^-$ γδ T cells, however this was not considered significant. There was also a trend towards a time x trial interaction of $CD94^-$ ($p=0.063$) however, when examining Figure 6.7.A, the biggest difference between trials appears to be during the morning priming bout of exercise. There was no significant difference in CD11a, CD62L, or CD94 expression of γδ T cells between the SIN and REP trials at any time point.

Exercise significantly increased in circulating concentrations of pDCs and total DCs in the immediately post-exercise period. Additionally, pDC concentration was significantly higher in the REP trial compared to the SIN trial at post-A.M.-Ex. However, no significant differences were observed in mDC, $CD16^+$ DCs, and total DCs between REP and SIN trials. There was however, a significant time x trial interaction present in tDCs and $CD16^+$

DCs. Previous research has reported that total DC concentration increases acutely following surgery, or a treadmill exercise test to exhaustion, with an equal rise in mDCs and pDCs [105]. Furthermore, immediately following a marathon run, mDC concentration increased while pDC concentration decreased [164]. On the other hand, following a 1-hour ice-hockey training session, both mDCs and pDCs increased significantly immediately after exercise [165]. The differences among these results may reflect varying sampling times after exercise. Additionally, differences in exercise intensity and duration may influence the cellular redistribution kinetics. As previous research has highlighted, significant changes in leucocyte redistribution can occur during the early stages of an exercise bout (30 min), but may return to near resting concentration after 2.5 h of exercise [155], due to the biphasic nature of the lymphocyte response to exercise [111]. Regardless, in the present study, pDCs appear to be responsible for the greatest change in total DC concentration due to exercise. Additionally, the significant time x trial interactions may be due to either the recovery period or the A.M.-Ex period. There appear to be differing recovery patterns occurring between SIN and REP trials, although they were not considered significantly different at any specific time point. The DC concentration kinetics appear to be different to be different between SIN and REP groups, although this may be due to the greater range of values seen post-exercise in the REP compared to the SIN group for both tDCs and $CD16^+$ DCs.

Concerning limitations of the present study, no stress hormone data was collected in the present study. While measuring the concentrations of cortisol, epinephrine and norepinephrine would be a luxury, it was not a primary aim of the research. Although hormonal analysis would provide further evidence to support the hypothesis that acute stress hormone increases are driving changes in cellular components, the cost of analysis would outweighed the value of the data collected. It would be expected that any stress hormone data would merely support countless previous research articles, rather than provide any new information. Indeed, studies of very similar duration and intensity, repeated 1.5 h bouts at ~75% $\dot{V}O_2$peak [177, 178], have previously examined these stress hormones, to expected and repeatable results. As such, it was decided not to analyse stress

hormone concentrations and rather complete more detailed analysis of cellular components of the immune system.

As noted above, many subsets (particularly total lymphocytes and its related subsets) featured near identical recovery patterns following P.M.-Ex between both trials. This may suggest that regardless of prior exercise, cellular redistribution follows a repeatable pattern following prolonged heavy exercise. These results are both novel and meaningful: our study suggests that prior exercise has less effect on cellular redistribution following a subsequent bout of prolonged heavy-intensity exercise than previously thought. We propose that this finding is possibly linked to the provision of adequate energy intake employed in the present study both prior to and in-between exercise sessions. This is important when considering the increased incidence of symptoms associated with URIs during periods of heavy training and competition [1-5]. It is during these times, that stress is at its greatest, and recovery is most important. This highlights the importance of maintaining carbohydrate stores, and ensuring adequate energy intake before exercise, not only for bioenergetics, but also for immune function.

7. DISCUSSION

This thesis examined the complex relationship between acute stress, exercise and immune function. The increased occurrence of upper respiratory illnesses during periods of heavy training, taper and competition is a cause for concern [8, 13, 14, 24-28]. Previous research in overtraining have observed URS were associated with significant persisting fatigue and poor performance [13-17]. Indeed, any interruption to optimal training is likely to impact an athlete's ability to train at the highest level. Closely linked to this repetitive training is the concept of stress. While exercise is one often studied stressor, many others exist which exhibit similar effects [104, 105].

Endurance athletes regularly undergo significant physiological stress caused by large training volumes. Training may occur multiple times per day, every day of the week, resulting in repetitive, stress-induced activation of the immune system. What appears to be the prominent question driving exercise immunology research in elite endurance athletes is: "What are the origins of the increased incidence of symptoms associated with URIs in these individuals?" Advances in research over the last 30 years has allowed a greater understanding of how the immune system functions and coordinates immune responses among the cells, proteins, and structures. When an individual is more or less susceptible to infection, what is causing this? Is it a single immune variable, cell type, protein, structure or otherwise that is compromising the entire integrity of a person's defence mechanisms? A compromise that can be exploited by an opportunistic pathogen. Or is it an overall decrease in holistic immune function which results in a greater chance of a pathogen entering the system and mounting a successful infection? In many diseases, it is very much the former. The human immunodeficiency virus (HIV) infects $CD4^+$ T cells, macrophages and dendritic cells, impairing the hosts' ability to function optimally in immune surveillance resulting in acquired immune deficiency syndrome (AIDS) [346]. Furthermore, those individuals presenting with Selective IgA Deficiency [49], and poor saliva flow rates (hyposalivation) [347], have been shown to have a high incidence of symptoms associated with URIs. In contrast to these two examples with a definite cause for a state of immunocompromise, stress-induced immune-dysfunction appears to be much more multifactorial. This is because the stress response is part of normal, healthy human

function [313]. Why then does this response sometimes cause a state of immunocompromise? And is the entire immune system affected, or is it a single or few small variables that are altered to the way of slight immune-dysfunction allowing the contraction of a URI? These are the questions that the area of exercise immunology as a whole are striving to answer, and are important to consider when looking to understand the complexity of this response.

One of the most important concepts throughout this thesis is the relationship between the stress response and subsequent immunological changes. Specifically, cellular redistribution following stress is paramount in understanding both the potentially positive and potentially negative effects of exercise on immune function. The influence of changes in circulating stress hormones and blood flow on cellular redistribution and function cannot be understated. It is important to remember, throughout this thesis, an increase in cell concentration of any leucocyte population in the blood following a stress response was defined as cellular ingress. Whereas, a decrease in cell concentration of any leucocyte population in the blood following a stress response was defined as cellular egress. Stress itself was defined as "*a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight or flight systems in the body (stress response)"* (reproduced with permission) [102, 103]. Specifically, this cellular ingress into peripheral circulation occurs by transportation of cells from areas of storage (i.e. spleen, lungs, marginated pool of respiratory tract, bone marrow, lymph nodes), into the circulation (i.e. blood vessels and lymphatics) and out to the tissues (i.e. skin, lung, gastro-intestinal and urinary-genital tracts, mucosal surfaces, and lymph nodes) [106, 107]. This stress response may (incidently) prepare immune cells to respond to opportunistic infectious pathogens [106, 108]. It has been suggested that the effect of stress on the immune system depends on the duration of the exposure to stress. Acute and intense stress may enhance immune function, and mild stress of moderate duration may promote immunosurveillance, while chronic stress may cause immune dysregulation [102, 109].

Referring to specific results of this thesis regarding cellular redistribution, one of the results that may be considered in contrast to previous findings was that no significant increase was present in circulating lymphocyte concentration following the 2 h cycle at 90% VT_2 in Study II. Immediately, this is in contrast to a large portion of previous research examining the cellular response to stress or exercise [1, 107, 315]. Indeed, when compared to Study IIIB, there is a distinct difference in the results immediately post-exercise in relation to the same cell types (total lymphocytes, total γδ T cells and subsets, total DCs and subsets). There would seem to be a disconnect between the two, as we observed a significant increase in lymphocyte concentration in Study IIIB compared to no change in Study II. This is particularly odd as all samples for both studies were analysed by the same haematology analyser undergoing regular and routine calibration (Coulter HmX Hematology Analyzer, Beckman Coulter). An alternative hypothesis for the difference between studies may be related to the duration of the bouts. Previously, during a 2.5-h treadmill run at 75 % of $\dot{V}O_2$peak, there was no significant change in lymphocyte concentration immediately post-exercise, while midway through the exercise bout, a significant increase occurred [155]. Although comparison between running and cycling can be risky due to the effects of eccentric loading on circulating leucocyte concentrations [348], one study examining lymphocyte concentrations in response to 2.5 h of running or cycling at ~75% VO_2peak (with and without carbohydrate supplementation) demonstrated no difference in lymphocyte concentrations during the post-exercise period, regardless of exercise mode, in experienced triathletes [196]. Potentially, a peak in ingress may occur during the exercise bout, but after 2 h of exercise, egress may be up-regulated enough to decrease circulating lymphocyte concentration (or cause it to appear unchanged) at the immediately post-exercise sampling point. This phenomenon has been previously observed in rats. After an initial increase in circulating concentration (ingress), a decrease occurs below resting concentrations (egress) while the stress stimulus (psychological stress in this case) is ongoing [106].

In addition to the differences between Studies II and IIIB in relation to circulating lymphocyte concentration immediately post exercise, dendritic cells and γδ T cells also demonstrated differences between the two studies. For γδ T cells, Study II demonstrated a

significant decrease in the concentration of γδ T cells and the δ2 subset up to 4 h post-exercise. Furthermore, phenotypical differentiation revealed a significant decrease in naïve and central memory δ2 cells. Additional analysis revealed preferential egress of γδ T cells expressing $CD11a^{high}$ and $CD62L^{high}$. Conversely, Study IIIB revealed significant exercise-induced increases in total lymphocytes, total γδ T cells, δ1 and δ2 subsets, and γδ T cells with high expression of CD11a, CD62L, and CD94. This was then followed by a decrease in the concentration of these subsets from 1 h post-exercise. Additionally, when comparing DCs, Study II showed a significant decrease in total DCs at 1 h post-exercise, together with decreases in mDCs and $CD16^{+}$ DCs up to 2 h post-exercise. Conversely, Study IIIB showed a significant immediate post-exercise increase in total DCs and pDCs followed by a decrease at 1 h post-exercise. As suggested above, these contrasting results between Study II and Study IIIB would appear to be related to the timing of sampling. This is highlighted by a similar pattern of circulating cell concentration changes during the post-exercise recovery period. Although, recovery points examined in the two studies aren't identical making direct comparison more difficult, changes in circulating DC concentration feature a decrease following exercise (egress) which returns to approximate resting concentration at 4 h or 6 h post-exercise. This appears to be consistent following both a single bout of exercise (Study II and Study IIIB), or repeated exercise (Study IIIB). These observations may suggest that cellular egress follows a repeatable pattern following exercise. Although it is clear that certain cell types are preferentially mobilised in response to stressors such as exercise [104], less is known about the process that occur during cellular egress.

It must be noted that the mitogen-stimulation assay was changed from Study I to Study IIIA. The original study used stimulated isolated PBMCs with PHA at a concentration of 1×10^6 cells/mL. This stimulation lasted for 72 h at 37°C. In contrast, Study IIIA involved a whole blood stimulation assay with PMA and ionomycin for 4 h at 37°C. Both these assays are used regularly in analysing immune function [155, 241, 242, 258, 349]. The reasons for the change of assay were two-fold: 1) in Study I, a high degree of variability between samples in a small cohort made analysis difficult, and; 2) stimulation with PMA and ionomycin was used previously when examining intracellular cytokine production of T

cells [155, 159], however, this stimulation cocktail has not been used to quantify cytokine production from whole blood. It was hoped the use of a different stimulation assay, that has proven effective in intracellular cytokine analysis, would provide further information that may have been missed in Study I. Unfortunately, due to the change in assay, comparing T helper cytokine concentrations between studies is impractical. This is because stimulation is via different mechanisms, on a different population of cells for a different duration. Examining the data of the Studies I & IIIA shows a large difference in concentrations of these different cytokines, with a different pattern of change among the seven cytokines that were analysed. Finally, magnitude-based inference analysis was employed in Study I but not Study IIIA. For Study I, this form of analysis was used as no significant changes at any time point for all cytokines analysed, however in Study IIIA significant changes and interactions were discovered using traditional statistics. Due to this, it was decided to not use magnitude-based inference for analysis Study IIIA, but rather a combination of ANOVA with repeated measures and correlation analysis.

Throughout this thesis, the immune recovery period is mentioned. By definition, this is referring to the period following the cessation of an acute stressor when cellular redistribution is occurring, until the point of where circulating cell concentration returns to resting levels. This is quite non-specific, primarily due to gaps in knowledge regarding the immune recovery period. This thesis aimed to uncover some of the changes that occur during this period. And indeed, previous research has highlighted the kinetics of certain changes, and the locations and directions certain cell types relocate to during this period [350-352]. But the question still remains concerning immune system recovery. In the muscle physiology, recovery is required to replete endogenous fuels sources, repair damaged muscle tissue, and produce proteins (such as enzymes and mitochondria), which will facilitate adaptation to a specific stressor and return to homoeostasis [353, 354]. Immune system recovery does not fit within the above terms. Other than the time period in which this occurs, there does not appear to be any similarities. So should we examine immune recovery the same way as muscle recovery? Some previous research has demonstrated short-term functional decreases in certain cell types. And indeed, there is a period of greater than eight hours for some cell types to return to resting concentrations [1]. But the issue lies in identifying a repeatable phenomenon driven by the exercise-induced

stress response which decreases host defence. Questions remain whether the increased occurrence of symptoms associated with URIs during periods of heavy training and competition related to a decreased ability to mount an immune response during this recovery period, rather than the "killing ability" of a single cell type.

A potentially important finding from this thesis is that of adequate energy intake prior to prolonged exercise. By no measure a new concept in exercise physiology and nutrition [355], less focus has been placed on energy intake in exercise immunology research. Although carbohydrate ingestion during exercise has been demonstrated to attenuate the rise in stress hormones (particularly cortisol) [196, 332], and more recent reviews have recommended carbohydrate as an imperative part of maintaining immune health with exercise [108, 356], many studies preferentially select fasted protocols during research, even during sampling periods of more than ten hours duration [186, 197, 357]. This choice is likely driven by the desire to remove the effect of food ingestion on circulating hormone concentrations. The issue is that this method provides an inaccurate representation of how the immune system is affected by exercise, both during, and following the exercise bout. It is recommended in exercise physiology that maintaining optimum blood glucose, and adequate glycogen stores are imperative to optimal performance, in training and competition [355]. In the present thesis, no 'carry-on' effect of prior exercise was evident in cell types other than neutrophils. Importantly, there was no significant difference between trials during afternoon exercise and recovery. It must be noted that there was no control group in which energy intake was controlled or limited to contrast with the energy matched protocol of the present studies. This makes reaching a conclusion difficult when considering the influence of energy intake on the potential 'carry-on' effect, however it does raise questions that could be answered in further research. Additionally, this may support the argument for normal recommended dietary intake to continue during exercise immunology research. Ensuring adequate energy intake in exercise immunology research may present an effect more demonstrative of the immunological changes that occur in athletes undergoing regular training, rather than an altered effect due to fasting or inadequate energy intake during/before exercise. The feeding protocol used in this thesis was decided on to reflect the eating habits of the highly trained cyclists who undertook the study. Specifically, it was deemed imperative that energy intake should match energy

expenditure to mirror training recommended protocols used by such highly trained athletes [358].

Another concept previously unexamined in exercise immunology literature is that of concentration groups. As mentioned above, the concept of sorting subjects into “high” and “low” and/or “non” responder groups has been used in research previously [323-327]. However, to our knowledge, no study in exercise and stress immunology has expressed changes in the immune system following an acute stressor by the resting concentration circulating cell subsets. As highlighted in Study II, in many cell subsets, those subjects with a higher resting cell concentration of a specific subset demonstrated a greater decrease in concentration during the egress phase, however this wasn’t consistent throughout, in particular total DCs. When examining cellular ingress into circulation of granulocytes and monocytes, resting concentration didn’t appear to influence peak concentrations. This may provide a basis for further research, as resting cell concentrations appear to be linked to magnitude of cellular egress post-exercise. It is recognised that elite athletes experience leucopenia driven primarily by neutropenia at rest [328, 329]. Potentially, these two findings may be related. Certainly, there is an interesting relationship between resting concentration of circulating leucocytes and the ability for these cell types to respond to stress. It has been hypothesised that an athlete’s stress response is more efficient than untrained due to regularly experiencing such efforts [329]; however, limited research has tested such a phenomenon.

Limitations

A number of limitations existed in the studies of this thesis. The lack of a non-exercising trial made parts of the analysis difficult in Studies I & II. In particular, Study II saw a number of changes during the post-exercise period which have not been seen before, and were in contrast to previous research. The reasons for the disparity between the present study and previous research may be due to the novelty of the variables analysed: both γδ T cells and DCs have rarely been measured in exercise immunology research. Furthermore, acute short-term stress (such as a psychological stress test) can drive different changes in the stress response and cellular redistribution compared to prolonged, heavy-intensity exercise. This makes drawing comparisons between studies more difficult. Regardless, a non-exercising trial may have provided a more solid foundation of which to contrast the findings against. In particular, the effects of diurnal variation could be separated from exercise-induced effects. As diurnal variation drives changes in plasma cortisol concentration, resulting in small changes in circulating leucocyte populations [309], it may have been advantageous to include a control trial. Although, the effects of diurnal changes are much smaller in magnitude compared to exercise-induced changes [177-179, 189, 197]. No non-exercising trial was included because one of the primary aims of the thesis was to acutely analyse the recovery period post-exercise. This required a large number of sampling points, which double when including a control trial. In previous research, the recovery period has been largely neglected, quite possibly due to the cost of analysing so many samples. This has become abundantly clear in the present studies. In the repeated bouts design, each subjects was sampled on sixteen occasions, multiplied by ten subjects and the cost of analysis using flow cytometry compounds very quickly. But the focus on the recovery period was one of the main aims of this research, and therefore, worthwhile.

Results from the mitogen stimulation assays (Studies I & IIIA) featured few significant changes between time points. Although mitogen stimulation is used widely in immunology research, contrasting results are often present between studies [96, 339]. Generally, these type of assays are used to analyse resting samples of individuals, in groups with quite pronounced immune deficiencies [243, 359, 360], although exercise immunology research has also used these analysis techniques [155, 159, 242, 250, 254-256]. In contrast, the current study was examining healthy subjects in an attempt to identify small changes in

immune function due to exercise. It would appear that exercise may not be drive large changes in cellular function, compared to a state of immune deficiency. As mentioned previously, two different stimulation assays were used. Study I employed at PHA-stimulation assay of isolated PBMCs, and Study IIIA used whole blood stimulation with PMA and ionomycin. Both these protocols are used in research regularly, however, may not have been adequate for the present research. Potentially, intracellular cytokine analysis may have been more useful. This involves a similar process to the present studies of mitogen stimulation, however additional steps are included where the cells undergoing analysis are bound to fluorescent monoclonal antibodies and permeated. Next, additional fluorescent monoclonal antibodies are added specific to the intracellular cytokines of choice. Flow cytometry is then used to examine production of cytokines by a specific cell type. Certainly, fewer time points and cytokines could be analysed compared to the present studies. However, previous research could have been used as a basis from which to design potential studies [155, 247]. These methods are more likely to be used for profiling cell types according to cytokine production, rather than cytokine production itself, however these results may be more useful in demonstrating the effects of exercise-induced stress on cellular immunity. Specifically, previous research has suggested the post-exercise decrease in T lymphocyte concentration is due primarily to a more pronounced decrease in Type 1 T cells (compared to Type 2 T cells). Potentially, intracellular cytokine production could be used to profile more cell types, such as dendritic cells which provide a large array of functions depending on their location current interactions, to better understand the specific response to stress during the post-exercise period. Ultimately, the goal is to determine if immune changes that occur with exercise can be linked directly with an increased risk of URIs.

In addition to changing certain assays, additional analysis would certainly have painted a more detailed picture, particularly when considering the more novel immune markers examined. Both γδ T cells and dendritic cells have been scarcely examined in previous research. In addition to the assays used in the present study, a functional analysis would be quite complimentary. Of course, one cannot analyse everything, but a functional assay would assist in discovering the acuities of these largely unexamined cell types. In this context, a functional tests analysing antigen-presenting capabilities, cytokines production

capabilities, or cytotoxic function could be meaningful. The primary difficulty, other than cost, would likely be related to very low circulating concentrations of DCs and γδ T cells. Potentially, an intracellular cytokine assay (such as that mentioned previously) would be the most effective and accurate measure of function. Regardless, both these cell types are of great interest; dendritic cells because of their role in coordinating immune responses; from recognition through to presentation and coordination of effector functions, or γδ T cells in their role as both an innate and adaptive cell located in high concentrations in the tissues, areas more at risk of potential infection.

For a thesis examining the stress responses effect on immune function, only Study I examined any form of stress hormone release. It was abundantly clear from the first study onwards that, while measuring the concentrations of cortisol, epinephrine and norepinephrine would be a luxury, it was not a primary aim of the research. Although hormonal analysis would provide further evidence to support the hypothesis that acute stress hormone increases are driving changes in cellular components, however the cost of analysis would outweighed the value of the data collected. It would be expected that any stress hormone data would merely support countless previous research articles, rather than provide any new information. Indeed, studies of very similar duration and intensity, 2 h at ~75% $\dot{V}O_2$peak (Study I & II) [197, 308], and repeated 1.5 h bouts at ~ 75% $\dot{V}O_2$peak (Study III) [177, 178], have previously examined these stress hormones, to expected and repeatable results. As such, it was decided not to analyse stress hormone concentrations and rather complete further analysis of cellular components of the immune system.

Finally, the participants recruited for this thesis were highly trained endurance cyclists. It is necessary to mention that results of the present study would not be identical to another athlete cohort (such as power or speed athletes) or a sedentary population. As highly trained endurance athletes undergo regular prolonged, heavy-intensity exercise-induced stress, their immune system would likely be more accustomed to dealing with such a stressor. Although few studies have directly compared cellular components of trained versus untrained individuals, one study demonstrated increased NK cell function in a group of highly trained cyclists [361]. Additionally, regular exercise has been showed to decrease

symptoms associated with URI compared to sedentary individuals [362]. Furthermore, elite swimmers undertaking intensive training have significantly lower neutrophil oxidative activity at rest than age- and sex-matched sedentary individuals, but URTI rates do not differ between the swimmers and sedentary controls [363]. As concluded on in a recent position statement “the nature of the reported changes measured depends on a number of variables that include the training status of the individual, the intensity of the exercise bout, and the nutritional status of the individual” [111]. This is important to consider when examining the results of the present study.

Recommendations & Future Directions

At the end of the day, the most important information to discover in the field of exercise immunology with elite athletes would be; 1) a single or combination of biomarkers that can accurately predict when an athlete is immunocompromised and at a greater risk of illness, and 2) a number of recommendations, interventions, and supplements that can be easily used by athletes to decrease the risk of contracting an illness. In the bubble of elite sport, these are the two major goals of research in this area. Previous research and practice of common sense allows the partial fulfilment of these goals. The most accurate biomarker currently in practice is sIgA. Functioning as part of the mucosal immune system, sIgA provides the 'first line of defence' against pathogens. To date, the majority of exercise studies have assessed saliva sIgA as a marker of mucosal immunity, but more recently the importance of other antimicrobial proteins in saliva (e.g. α-amylase, lactoferrin and lysozyme, cathelicidin antimicrobial peptide, human neutrophil alpha-defensins, human beta-defensin 2, chromogranin A) has gained greater recognition [364-373]. Depressed secretion of sIgA into saliva during periods of intensified training and chronic stress are likely linked to altered activity of the hypothalamic-pituitary-adrenal axis, with inhibitory effects on IgA synthesis and/or transcytosis [5, 26, 374, 375]. Consensus exists that reduced levels of saliva sIgA are associated with increased risk of URI during heavy training [111]. Additionally, sIgA is used in many professional sporting teams through the sporting season as a monitoring tool. Regarding potential recommendation, interventions and supplements to maintain immune health, the position statement by published by a consortium in Exercise Immunology Review has provided the "gold standard" for dealing with illness in athletes [108], in addition to nutritional considerations which are of high importance [356]. Ensuring adequate energy, carbohydrate and protein intake and avoiding deficiencies of micronutrients are key to maintaining immune health. Evidence is accumulating that some nutritional supplements such as quercetin and probiotics can augment some aspects of immune function and reduce illness rates in exercise stressed athletes [244, 376, 377]. Of course, prevention of infection is always superior to treatment. Although there is no single method that completely eliminates the risk of contracting an infection, there are several effective methods of reducing the risk of infection. These means include appropriate management of training loads, use of appropriate recovery strategies, good personal hygiene, avoiding contact with large crowds, young children and sick

people, good nutrition, getting adequate good quality sleep and limiting other life stresses to a minimum [108].

Regarding findings of the present thesis, the following recommendations have been suggested regarding specifics markers examined and the protocols used. Repeated exercise appeared to have less of an effect on cellular mobilization that previously observed. Although a firm conclusion isn't possible, this may be due to energy intake during the testing period. Adequate nutrition is clearly important in maintaining immune health, perhaps when designing protocols in the future energy expenditure and consumption should be matched to more accurately reflect conditions predominantly seen in real life, rather than fasting. Furthermore, repeated exercise may be less detrimental to immune health than previously thought, provided that adequate energy intake in completed specifically in trained individuals. The present study decided to employ an exercise protocol determined by a percentage of the second ventilatory threshold, rather than a percentage of VO_2max. This was to allow a more accurate representation of exercise intensity that would limit the effects of oxygen consumption and cardiac drift, which would in turn affect circulating stress hormone concentrations. A large proportion of previous research in exercise immunology has determined exercise intensity as a percentage of maximum. This is dangerous, as each individuals thresholds differ, as does their perception of the exercise effort. By calculating exercise intensity according to the anaerobic or second ventilatory threshold (or potentially critical power), a much more standardised mark would be set, applicable across all participants regardless of training status. Regarding training status, the present study elected to examine highly trained cyclists. This made recruitment very difficult as strict recruitment criteria were used to ensure an elite level group. This was important when examining the effects of repeated exercise-induced stress on a population that regularly undertakes such efforts in Study III. However, in Study I and Study II it may have been much easier (and likely to demonstrate similar results), if a recreationally active healthy population was used. Recruitment can be one of the most difficult parts of research, made much more difficult by recruiting a rare population group that is required to give up time, energy and blood in the name of research. Finally, regarding cellular immune markers and mitogen-stimulated cytokine release, while some meaningful results were found, the way forward in exercise immunology research may be a

more holistic approach. Of course, there is no better simulator of the effects of elite level training, than completing a training study on elite level athletes. In order to identify a biomarker which could be used to identify when an athlete is immunocompromised, a study on an elite population throughout a season is likely the way to discover this. A holistic approach would be necessary to understand the complexities of the immune system under different forms of stress. Surveys such as daily analysis of life demands for athletes (DALDA) [378], profile of mood states (POMS) [379], and quantification of training load can be used to understand life stressors. Simple, less invasive techniques like saliva collection can be used to analyse an array of measures such as SIgA, cortisol, α-amylase, lactoferrin and lysozyme, cathelicidin antimicrobial peptide, human neutrophil alpha-defensins, human beta-defensin 2, and chromogranin A. Blood collection to analyse hormones, cytokines, proteins, and cells of the immune system; both at rest and during exercise may be used. Additionally, immunological stress tests such as delayed-type hypersensitivity response could be used to assess immune function. Furthermore, URI episodes should be properly defined and medically verified. Such a research undertaking may be required to identify a single, or combination of variables that are truly indicative of compromised host defence.

Conclusion

This thesis aimed to examine the immune system response and subsequent recovery period following prolonged heavy-intensity exercise in highly trained individuals. Specifically, to analyse the effect of single and repeated bouts of prolonged heavy-intensity exercise on a number of novel markers of immune system function including, dendritic cells, γδ T cells, and T helper cytokines with focus on both the immediate exercise effect and the subsequent immune recovery period after exercise. The results of the present thesis were planned to be two-fold; 1) expand the knowledge base for a number of novel immune markers previous unexamined under severe, prolonged stress stimuli, and 2) provide further evidence of how repeated immune stimulation may be detrimental to healthy immune function when recovery between sessions is not sufficient. Qualitative analysis revealed post-exercise changes in concentrations of plasma cortisol, IL-2, TNF, IL-4, IL-6, IL-10, and IL-17A compared to pre-exercise values. A Th1/Th2 shift was evident immediately post-exercise. Furthermore, for multiple cytokines including; IL-2 and TNF (Th1), IL-6 and IL-10 (Th2), and IL-17 (Th17) no meaningful (magnitude-based inference) change in concentration occurred until more than 4 hours post-exercise, highlighting the duration of exercise-induced changes in immune function. Study II demonstrated a significant decrease in the circulating concentration of γδ T cells and the δ2 subset up to 4 h post-exercise. Phenotypical differentiation revealed a significant decrease in circulating naïve and central memory δ2 cell concentrations. Additional analysis revealed preferential egress out of circulation of γδ T cells expressing $CD11a^{high}$ and $CD62L^{high}$. Finally, a significant decrease was evident in circulating total dendritic cell concentration at 1 h post-exercise, together with decreases in circulating myeloid dendritic cells and $CD14^{-/low}CD16^{+}$ dendritic cells up to 2 h post-exercise. Additionally, Study IIIA showed significant increases in stimulated cytokine concentrations (compared to pre-exercise) of Th1 cytokine; IL-2, and Th2 cytokines; IL-4 and IL-10. When adjusted for total leucocyte concentration at each time point, a significant increase from pre-exercise was observed in Th1 cytokines; IL-2, TNF, and IFN-γ, and Th2 cytokines; IL-4 and IL-10. However, there were no significant differences between the single and repeat exercise bout trials at any time point. Finally, Study IIIB revealed a significant exercise-induced increase in circulating concentrations of total lymphocytes, neutrophils, total γδ T cells, δ1 and δ2 subsets, total dendritic cells, and the plasmacytoid dendritic cells. Furthermore, among the

γδ T cells, those with high expression of CD11a and CD62L were preferentially mobilised. However, no significant differences were observed between the single and repeated exercise trials for any variable, except total circulating lymphocyte and neutrophil concentrations. It appears the second aim resulted in some unexpected effects. Primarily, that repeated exercise did not result in a "carry-on" effect from the first exercise bout to the second may suggest repeated exercise with minimal recovery may not be as detrimental to cellular components of immune function as previously thought, provided there is adequate energy intake between sessions. Together, these results highlight the multifactorial nature of both; the stress response, and the immune system with its complex interactions of proteins, cells and structures functioning together to defend the host against invading pathogenic agents.

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APPENDICES

A. Supplementary Flow Cytometry Information – γδ T cells

δ1/δ2 T cells and memory phenotypes

Total lymphocytes were gated using forward versus side scatter (Figure A.1.A). δ1 T cell were defined as $CD3^{+}V\delta1^{+}$ lymphocytes and δ2 T cells as $CD3^{+}$ $V\delta2^{+}$ lymphocytes (Figure A.1.B). δ1 and δ2 T cells were further defined as NA, CM, EM, or EMRA according to expression of CD27 and CD45RA (Figure A.1.D). As a comparison, $CD3^{+}$ lymphocytes differentiated according to expression of CD27 and CD45RA is displayed in (Figure A.1.C). Lymphocyte subset numbers were quantified using the percentage values obtained from each lymphocyte subset gate. The absolute lymphocyte concentration was obtained from the full blood count.

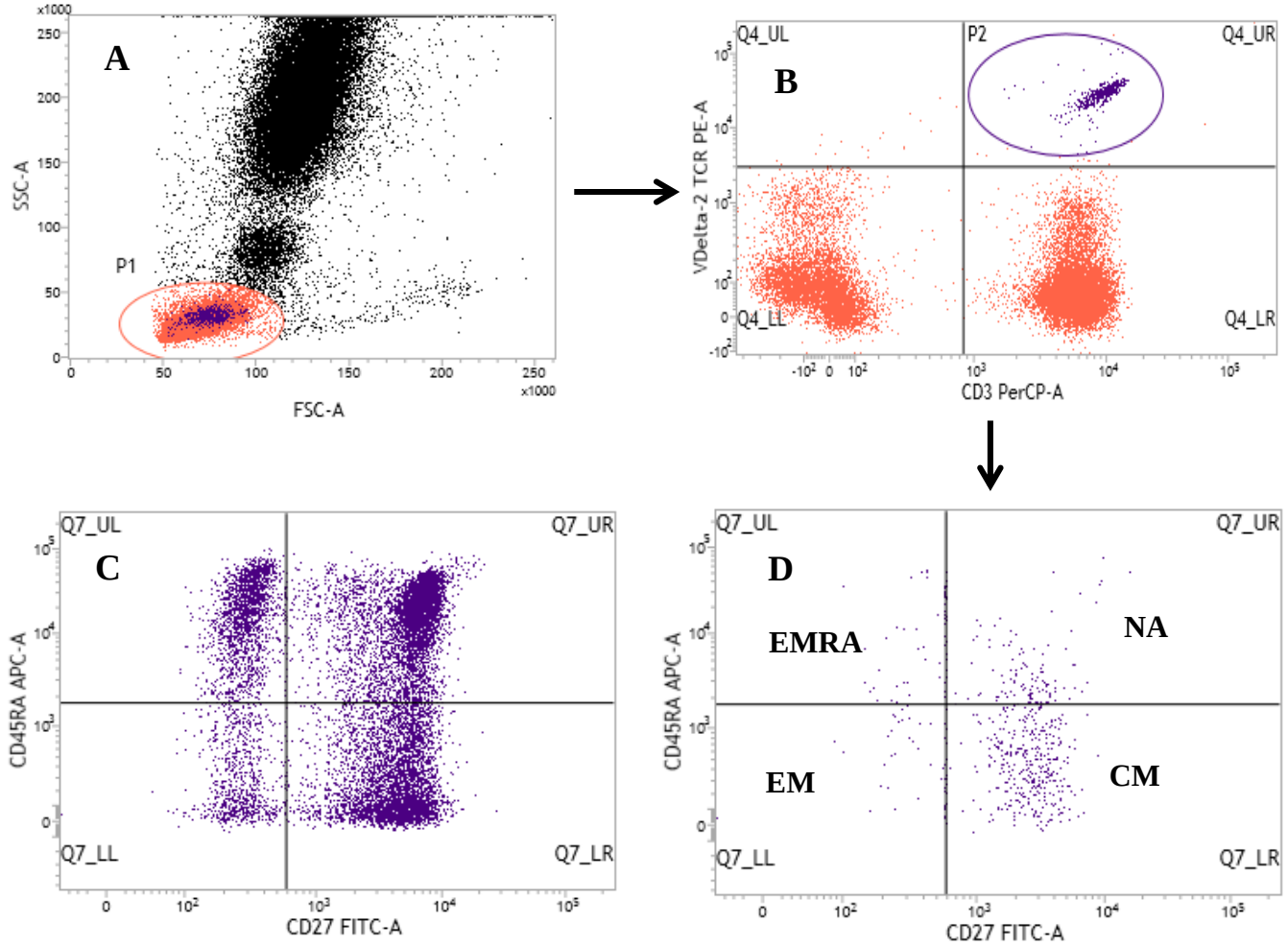

Figure A.1. Identification of δ1 and δ2 T cell memory phenotypes. A: Forward vs. side scatter of lysed whole blood. B: CD3 vs. Vδ2 TCR of gated lymphocyte population. C: CD27 vs. CD45RA of $CD3^+$ gated lymphocyte population. D: CD27 vs. CD45RA of $CD3^+$/ $V\delta2^+$ lymphocytes.

Total γδ T cells and CD11a, CD94, and CD62L expression

Total γδ T cells were gated as $CD3^+$ γδTCR^+ (Figure A.2). These cells were then examined for expression of CD62L (Figure A.3.A) and (Figure A.3.D), CD11a (Figure A.3.B) and (Figure A.3.E), and CD94 (Figure A.3.C) and (Figure A.3.F). The gated lymphocyte population expressing CD3 was used to determine expression of CD11a (low/high), CD62L (low/high), and CD94 (-/+). These gates were then applied to $CD3^+$ γδ TCR^+ cells.

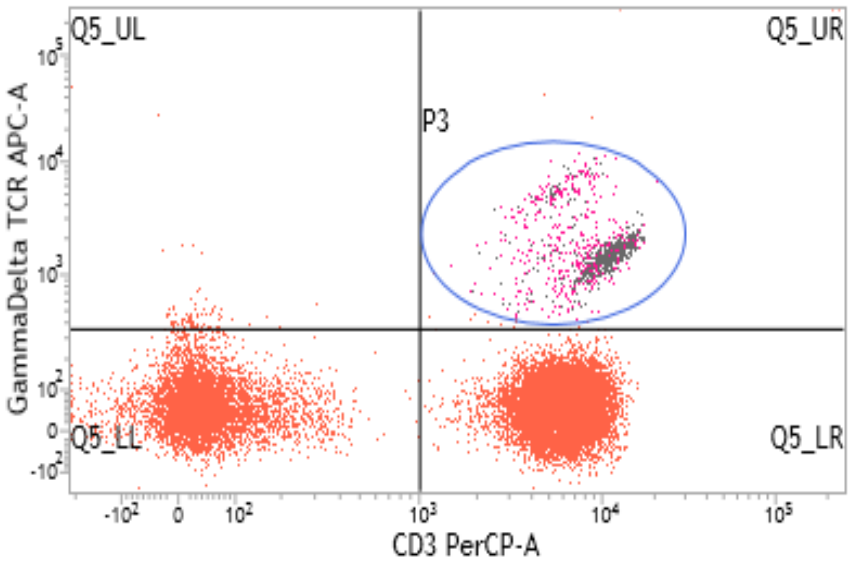

Figure A.2. Identification of total γδ T cells. CD3 vs. γδ TCR of gated lymphocyte population

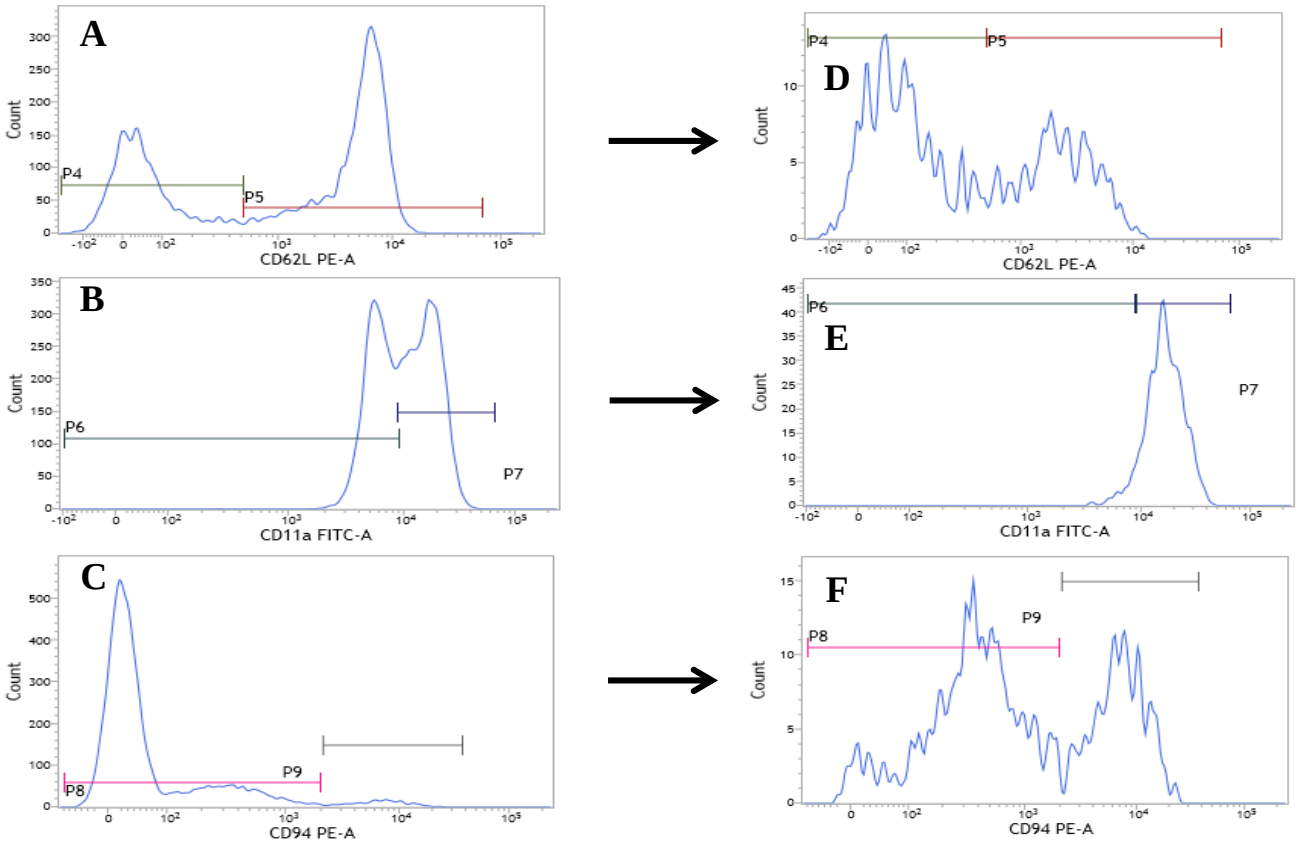

Figure A.3. Surface expression of CD11a, CD94, and CD62L. A: CD62L expression of $CD3^+$ lymphocytes. B: CD11a expression of $CD3^+$ lymphocytes. C: CD94 expression of $CD3^+$ lymphocytes. D: CD62L expression of $CD3^+/\ \gamma\delta^+$ lymphocytes. E: CD11a expression of $CD3^+/\ \gamma\delta^+$ lymphocytes. F: CD94 expression of $CD3^+/\ \gamma\delta^+$ lymphocytes.

B. Supplementary Flow Cytometry Information – Dendritic Cells

Expression of CD33 and HLA-DR and absence of the lineage cocktail 2 with intermediate forward (FSC) and side scatter (SSC) between those of lymphocytes and monocytes was used to determine tDCs (Figure B.1.A) and (Figure B.1.B). Dendritic cells were further defined into three subpopulations:

- mDC ($CD123^{dim}$/$CD33^{high}$/$CD16^{-}$) (Figure B.1.C)
- $CD14^{-/low}CD16^{+}$ DC ($CD123^{inter}$/$CD33^{inter}$/$CD16^{+}$) (Figure B.1.D)
- pDC ($CD123^{high}$/$CD33^{-/dim}$/$CD16^{-}$) (Figure B.1.E)

Absolute counts were quantified using percentage values obtained from each lymphocyte and monocyte subset gate.

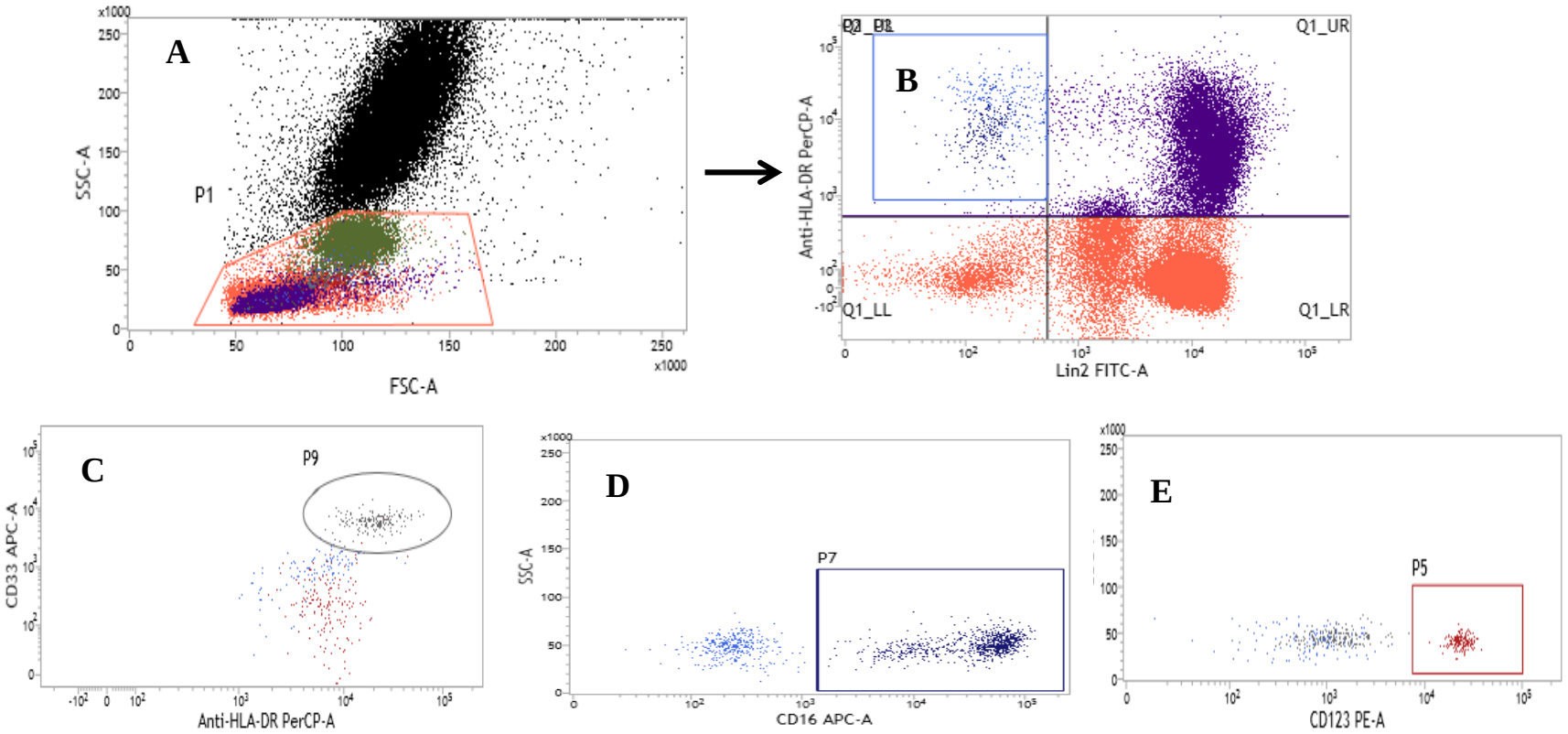

Figure B.1. Identification of total dendritic cells and subpopulations. A: Forward vs. side scatter of lysed whole blood. B: Lineage cocktail vs. HLA.DR of gated lymphocyte/monocyte population. C: CD33 expression of $Lin^-/HLA.DR^+$ gated population. . D: CD16 expression of $Lin^-/HLA.DR^+$ gated population. E: CD123 expression of $Lin^-/HLA.DR^+$ gated population.

C. Explanatory Statement – RO955 (2009) – Study I

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Explanatory Statement

The effects of sub-maximal exercise on acute immunological function

BUHREC Protocol Number: RO-955

Purpose:

Determination of Maximum Oxygen Consumption (VO_{2max}) and immune markers during 2hrs at 70% VO_{2max}

Investigator's contact details:

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Ethics officer details:

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Where will the testing be conducted?

Our testing will be conducted in the Faculty of Health Sciences and Medicine Building at Bond University. This building is located on the southern side of the campus. The Exercise-Testing Laboratory is on the ground floor.

Directions as follows:

1. Turn into University Drive.
2. Turn right at the second round-a-bout.
3. Take the first left turn into the service road (Western side of University).
4. Park one of the bays directly outside the Faculty of Health Sciences and Medicine building.
5. Reception is on level 1 (Ground Floor) in the middle of the building.

Change rooms with showers are available close to the area where the exercise testing program will be conducted.

Overview of the testing and pre-testing

The VO_{2max} Test

Prior to the test

Once you arrive you will be taken through the Explanatory Statement, PAR-Q, Health and Training Questionnaires in which you will sign a consent form once all your questions have been answered. You will have a 12 lead ECG attached to your chest and will be seated.

Note: At any time you may asked for the test to be stopped and have no obligation to continue with any testing.

Your VO_{2max} Test

The VO_{2max} test will be conducted on a stationary cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by a qualified exercise physiologist. No blood sampling will occur during this test. You will have a 12 lead ECG attached to your chest and will be seated with your ECG monitored. You will then be invited to sit on the stationary bike at which time you will be able to warm up (relaxed pedalling on a light resistance). Following the warm up a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip) to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready in which you will commence the protocol below.

- Warm Up - 6 min at 80W.
- Test - Increase 10W every 20 sec.
- Warm Down - 50W

You will remain cycling on the stationary bike to the point at which you reach maximum oxygen output. Following reaching maximum you will be instructed to warm down while your ECG is continuously monitored. You may withdraw from the test at any time.

Following Exercise

Once you have completed the VO_{2max} test you will be seated or directed to a bed to recover and be continually monitored. After 30min you are able to return home following a health check questionnaire to ensure you are safe to leave the premises.

Health risks associated with a max test

With the right preparation, the VO_{2max} test is a safe procedure. However, even with the right preparation there is a risk that a misadventure could occur. The misadventure may include falling over, dizziness, heart attack and death. The American College of Medicine states, "exercise only provokes cardio-vascular (heart) events in individuals with pre-existing heart disease. Exercise does not provoke cardiac (heart) events in individuals with normal cardiovascular systems".

When you arrive at the Exercise Testing Laboratory at Bond University you will be asked to complete a general health questionnaire and a training questionnaire. Additionally a resting 12 Lead Electrocardiogram and resting blood pressure will be taken.

These pre-exercise-screening procedures are intended to prepare you for the VO_{2max} test and to ensure your safety and well-being during the exercise test.

Exercise related cardiac events in adults

The overall risk of exercise testing in a mixed subject population is approximately 4-6 cardiac events per 10,000 exercise tests. Cardiac events include heart attack, very rapid and irregular heartbeats and death.

With the correct pre-screening (preparation) before the VO_{2max}, and with your heartbeat and heart rhythm closely monitored during the VO_{2max} test by an experienced exercise physiologist, the risk of cardiac misadventure is small.

Our strategies to minimize risk of a cardiac event

We will minimize the risk of a cardiac (heart) event during your VO_{2max} test and 2hrs at 70% VO_{2max} Test by asking you to complete the following;

1. A modified physical activity readiness questionnaire (PAR-Q). The PAR-Q is a medical questionnaire which provides preliminary information about your health.

2. A medical questionnaire detailing your medical history, your family history, your smoking, drinking, and physical activity habits. You will also be asked about any drugs you may take.
3. A training questionnaire detailing your training habits of the past year.

The 2hrs at 70% VO_{2max} Test

On the day of the test

Once you arrive, you will have a 12 lead ECG attached to your chest and will be seated. After 15 minutes you will have a blood sample taken from an antecubital vein, where a total of 45mL of blood will be taken. Blood will be taken on 7 occasions, 45mL each time. A 10min passive drool will also be taken pre-exercise and at each of the blood sampling points.

Your 2hrs at 70% VO_{2max} Test

The 2hrs at 70% VO_{2max} Test will be conducted on a stationary cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by a qualified exercise physiologist. Blood sampling will occur pre-exercise, post-exercise, 2hr, 4hrs, 6 hrs, 8 hrs, and 24hrs post-exercise (see figure 1).

Total number of blood/saliva samples = 7 samples.

Figure 1 – Blood Sampling during 2hrs at 70% VO_{2max} Test

Following Exercise

Once you have completed the 2hrs at 70% VO_{2max} you will be seated or directed to a bed to recover and be continually monitored. Between the sampling times of 2hr, 4hrs, 6hrs, and 8hrs you will be allowed to move around within the confines of the confines of the testing area.

Following the sample at 8hrs you are able to return home following a health check questionnaire to ensure you are safe to leave the premises. If you do not live close to Bond University you may chose to stay overnight on Gold Coast (nearby the university) free of charge.

A call by the Honours Student/PI at approximately 8hrs post-exercise will be made to check on you. You are required to return for a final sample to be taken 24hrs post-exercise, also you will be required to answer a health check questionnaire to ensure it is safe for you to leave the premises.

Passive Drool

A passive drool will be taken at every sampling point (n=7). This 10 min passive drool will be used to test for salivary immunoglobulin A. This can be quite an awkward process and thus it is recommended you follow these instructions to obtain the best and most efficient sample.

Prior to Collection:

- Do not eat a major meal within 60 min prior to sample collection.
- Avoid alcohol consumption 24 hours prior to sample collection.
- Avoid eating dairy productions during the 30 min prior to sample collection.
- Avoid acidic (eg. Cola drinks) or high sugar foods and drinks (eg. Confectionary, sports drinks, or soft drinks) during 60 min prior to sample collection.
- Do not brush your teeth within 2 hours of sample collection.
- Wash your mouth out with water 10 min prior to giving sample.

Saliva Sample Collection:

- The collection procedure will involve a 10 min sample collection period. Passive drool spit will be collected though a short plastic straw into a 5 ml vial.
- To effectively provide a sample of your salivary drool you should allow saliva to "pool" in your mouth and then without spitting, open your mouth to drool down through the straw into the collection vial with your head tilted forward.

- This method can be repeated as often as necessary until a sufficient sample of saliva is collected.
- Collection tubes will be weighed before and after sample collection to determine flow rate and volume.

Benefits from you participation

You will receive a substantial amount of information about your physiological and immunological response to exercise. We will explain the implications of all results which include; VO_{2max}, T- and Natural killer lymphocyte numbers, natural killer cell lysis activity, TH_1/TH_2, erythrocyte aggregation and deformability, neutrophil phagocytic function and respiratory burst capacity.

Information about the results of your test

Who will use the information from your results?

The results of your test will be confidential and contained within the people conducting the exercise test.

Do I get a copy of my results and feedback on participation?

You will receive collective results of the study of which the implication will be explained to you. This also provides an opportunity for you to ask questions.

D. Explanatory Statement – RO955 (Amended 2011) – Study II

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Explanatory Statement

The effects of sub-maximal exercise on acute immunological function

BUHREC Protocol Number: RO-955

Purpose:

Determination of Maximum Oxygen Consumption ($\dot{V}O_2max$) and immune markers during 2 h at 70% $\dot{V}O_2max$

Investigator's contact details:

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Dr Michael Simmonds
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Faculty of Health Sciences and Medicine, Bond University

Ethics officer details:

Caroline Carstens
Bond University Research Ethics Committee (BUHREC)
Email: buhrec@bond.edu.au
Phone: 5595 4194

Where will the testing be conducted?

Our testing will be conducted in the Faculty of Health Sciences and Medicine Building at Bond University. This building is located on the southern side of the campus. The Exercise-Testing Laboratory is on the ground floor.

Directions as follows:

1. Turn into University Drive.
2. Turn right at the second round-a-bout.
3. Take the first left turn into the service road (Western side of University).
4. Park one of the bays directly outside the Faculty of Health Sciences and Medicine building.
5. Reception is on level 1 (Ground Floor) in the middle of the building.

Change rooms with showers are available close to the area where the exercise testing program will be conducted.

Overview of the testing and pre-testing

The Familiarisation Session

Once you arrive the Explanatory Statement, PAR-Q, Health and Training Questionnaires will be explained to you. If you still wish to participate, we will ask that you sign a consent form once all your questions have been answered.

Lung function will be examined using a spirometer where forced vital capacity (FVC) will be tested to rule out pulmonary disorders. A finger prick blood sample will be taken to test for cholesterol and glucose. Additionally, if cholesterol is found to be outside the normal range, further blood sample will be taken for blood lipids to be tested.

You will have a 12 lead ECG attached to your chest that will be monitored while you are seated for 15 minutes. Additionally, supine/standing blood pressure will be taken.

Note: At any time you may asked for the test to be stopped and have no obligation to continue with any testing.

The Familiarisation session will be conducted on a stationary cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by an exercise physiologist with extensive experience testing individuals with chronic disease and high-level athletes. You will then be invited to sit on the stationary bike at which time you will be able to warm up (relaxed pedalling on a light resistance). Following the warm up a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip) to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready. During the test, you will cycle against a series of increasing resistance levels (at 30W per minute). You will continue cycling on the stationary bike until the point your heart rate reaches 120 beats/minute. Following reaching the resistance level associated with 120 beats/minute, you will be instructed to warm down while your ECG and blood pressure are monitored. You may withdraw from the test at any time.

This session is used to get you accustomed to wearing the oxygen mask and nose clip while exercising, to ensure you reach your maximal possible output during the $\dot{V}O_2$max test.

Following Exercise

Once you have completed the familiarisation you will be seated or directed to a bed to recover and be continually monitored. After 15 min you are able to return home following a health check questionnaire to ensure you are safe to leave the premises.

The $\dot{V}O_2$max test will be at least 12 hours after the familiarisation.

The $\dot{V}O_2$max Test

Prior to the test

Once you arrive you will be taken through the PAR-Q again to ensure you are safe to exercise. You will have a 12 lead ECG attached to your chest and will be seated for 15 minutes. Blood pressure will also be taken.

Note: At any time you may asked for the test to be stopped and have no obligation to continue with any testing.

Your $\dot{V}O_2$max Test

The $\dot{V}O_2$max test will be conducted on a stationary cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by an exercise physiologist. You will then be invited to sit on the stationary bike at which time you will be able to warm up (relaxed pedalling on a light resistance at 80W). Following the warm up a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip) to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready in which you will cycle against a series of increasing resistance levels (30W per minute) while cycling on the stationary bike to the point at which you reach maximum oxygen output. Your blood pressure will be taken at the approximate midpoint of the test. This test lasts approximately 15 min. Following reaching maximum you will be instructed to warm down while your ECG and blood pressure are monitored. You may withdraw from the test at any time. At all times during the test you must remain seated.

You may terminate the test at any time. Alternatively, we may terminate your test if:

- Adverse changes in your ECG and/or blood pressure are seen
- Your cadence drops 15 rpm below your preferred cadence

Following Exercise

Once you have completed the $\dot{V}O_2$max test you will be seated or directed to a bed to recover and be continually monitored. After 30 min you are able to return home following a health check questionnaire to ensure you are safe to leave the premises.

Health risks associated with a max test

With the right preparation, the $\dot{V}O_2$max test is a safe procedure. However, even with the right preparation there is a risk that a misadventure could occur. The misadventure may include falling over, dizziness, heart attack and death. The

American College of Medicine states, "exercise only provokes cardio-vascular (heart) events in individuals with pre-existing heart disease. Exercise does not provoke cardiac (heart) events in individuals with normal cardiovascular systems".

When you arrive at the Exercise Testing Laboratory at Bond University you will be asked to complete a general health questionnaire and a training questionnaire. Additionally a resting 12 Lead Electrocardiogram and resting blood pressure will be taken.

These pre-exercise-screening procedures are intended to prepare you for the VO_2max test and to ensure your safety and well-being during the exercise test.

Exercise related cardiac events in adults

The overall risk of exercise testing in a mixed subject population is approximately 4-6 cardiac events per 10,000 exercise tests. Cardiac events include heart attack, very rapid and irregular heartbeats and death.

With the correct pre-screening (preparation) before the VO_2max, and with your heartbeat, heart rhythm, and blood pressure closely monitored during the VO_2max test by an experienced exercise physiologist, the risk of cardiac misadventure is small.

Our strategies to minimize risk of a cardiac event

We will minimize the risk of a cardiac (heart) event during your VO_2max Test and Repeated 1 h Steady State Exercise Tests by continuously monitoring you ECG and blood pressure, and by asking you to complete the following;

4. A modified physical activity readiness questionnaire (PAR-Q). The PAR-Q is a medical questionnaire which provides preliminary information about your health.
5. A medical questionnaire detailing your medical history, your family history, your smoking, drinking, and physical activity habits. You will also be asked about any drugs you may take.
6. A training questionnaire detailing your training habits of the past year.

The 2 h at ~70% VO_2max Test

(This session will occur on TWO occasions – both Placebo and Carbohydrate supplementation during exercise)

Before the test

We ask you not to exercise at a high intensity within 2 days before the 2 hour test. Furthermore, we would like you not to exercise at all the day before the 2 h cycle test as this will have implications on your immune system.
We will provide you with a list of food options to eat the night before to ensure you ingest enough carbohydrates, protein and fat for the experimental trial.

We will also ask you to keep a simple training and illness log for a month, which will be explained by the researcher.

Please abstain from caffeine and alcohol for the 24 h before the test.

On the day of the test

On the day of the test, please do not eat breakfast. We will take your resting blood and saliva sample and then you will be fed before you begin exercising on the bike. You will then have a 12 lead ECG attached to your chest and will be seated for 15 minutes.

Your 2 h at ~70% VO_2max Test

The 2 h at ~70% VO_2max Test will be conducted on a stationary cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by a qualified exercise physiologist. A carbohydrate drink or placebo will be fed to you during the exercise. Blood sampling will occur pre-exercise, post-exercise, 1 h, 2 h, 4 h, and 6 h post-exercise (see figure 1). Total number of blood/saliva samples = 7 samples.

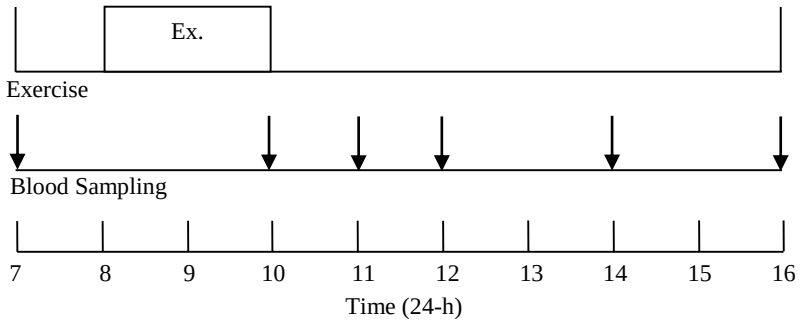

Figure 1 – Blood Sampling during 2 h at 70% VO_2max Test

Following Exercise

Once you have completed the 2 h at ~70% VO_2max you will be seated or directed to a bed to recover and be continually monitored. Between the sampling times of 1 h, 2 h, 4 h, and 6 h you will be allowed to move around within the confines of the testing area. Meals will be provided throughout this period.

Following the sample at 6 hrs you are able to return home following a health check questionnaire to ensure you are safe to leave the premises.

Passive Drool

A passive drool will be taken at every sampling point (n=7). This 10 min passive drool will be used to test for salivary immunoglobulin A. This can be quite an awkward process and thus it is recommended you follow these instructions to obtain the best and most efficient sample.

Prior to Collection:

- Do not eat a major meal within 60 min prior to sample collection.
- Avoid alcohol consumption 24 hours prior to sample collection.
- Avoid eating dairy productions during the 30 min prior to sample collection.
- Avoid acidic (e.g. Cola drinks) or high sugar foods and drinks (eg. Confectionary, sports drinks, or soft drinks) during 60 min prior to sample collection.
- Do not brush your teeth within 2 hours of sample collection.
- Wash your mouth out with water 10 min prior to giving sample.

Saliva Sample Collection:

- The collection procedure will involve a 10 min sample collection period. Passive drool spit will be collected though a short plastic straw into a 5 ml vial.
- To effectively provide a sample of your salivary drool you should allow saliva to "pool" in your mouth and then without spitting, open your mouth to drool down through the straw into the collection vial with your head tilted forward.
- This method can be repeated as often as necessary until a sufficient sample of saliva is collected.
- Collection tubes will be weighed before and after sample collection to determine flow rate and volume.

Benefits from you participation

You will receive a substantial amount of information about your physiological and immunological response to exercise. We will explain the implications of all results which include; VO_2max and threshold data, Natural killer lymphocyte numbers, natural killer cell lysis activity, TH_1/TH_2/Th_{17} cytokines, neutrophil phagocytic function and respiratory burst capacity and a full blood count. Furthermore, preliminarily blood glucose and blood lipid measurements will be taken to measure your risk of cardiovascular and metabolic diseases.

Information about the results of your test

Who will use the information from your results?

The results of your test will be confidential and contained within the people conducting the exercise test.

Do I get a copy of my results and feedback on participation?

You will receive your personal results and the collective results of the study of which the implication will be explained to you. This also provides an opportunity for you to ask questions.

E. Explanatory Statement – RO1145

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Explanatory Statement

Immune responses to repeated bouts of exercise

BUHREC Protocol Number: RO-1145

Purpose:

Determination of Maximum Oxygen Consumption (VO_{2max}) and measurement of immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests)

Investigator's contact details:

Supervisor: Associate Professor Sonya Marshall-Gradisnik
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Ethics officer details:

Caroline Carstens
Bond University Research Ethics Committee (BUHREC)
Email: buhrec@bond.edu.au
Phone: 5595 4194

Where will the testing be conducted?

Our testing will be conducted in the Faculty of Health Sciences and Medicine Building at Bond University. This building is located on the southern side of the campus. The Exercise Testing Laboratory is on the ground floor.

Directions as follows:

1. Turn into University Drive.
2. Turn right at the second round-a-bout.
3. Take the first left turn into the service road (Western side of University).
4. The Faculty of Health Sciences and Medicine is located 300m down the service road on the left.
5. Reception is on level 1 (Ground Floor) in the middle of the building.

Change rooms with showers are available close to the area where the exercise testing program will be conducted.

Overview of the testing and pre-testing

Prior to Testing

On each testing occasion you will be required to bring a number of items:

- Towel
- Water bottle
- Cycling attire
- Pedals
- Cycling shoes/cleats

Note: We have a set of Shimano pedals and a set of Look pedals, so if you have Shimano cleats you do not need to bring in your own pedals, however if you have another brand of pedals (e.g. Time, Speedplay, etc.) you will be required to bring them with you.

In the lead up to any of the exercise tests you should avoid the following:

- Alcohol the day before the test

- Caffeine the day of the test
- A large meal in the 3 hours before the test
- Heavy exercise the day before the test

The Familiarisation Session

Once you have arrived the Explanatory Statement, Physical Activity Readiness Questionnaire, Medical History and Training Questionnaires will be explained to you. If you still wish to participate, we will ask that you sign a consent form once all your questions have been answered.

Lung function will be examined using a spirometer where forced vital capacity (FVC) will be tested to rule out pulmonary disorders. A finger prick blood sample will be taken to test for cholesterol and glucose. Additionally, if cholesterol is found to be outside the normal range, further blood sample will be taken for blood lipids to be tested. Next, your height and body mass will be taken.

You will have a 12 lead ECG attached to your chest that will be monitored while you are resting for 15 minutes. Additionally, supine/standing resting blood pressure measurements will be taken.

Note: At <u>any time</u> you may asked for the test to be stopped and have <u>no obligation to continue</u> with any testing.

The Familiarisation session will be conducted on a cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by an exercise physiologist with extensive experience testing individuals with chronic disease and high-level athletes. You will be invited to sit on the cycle ergometer at which point another blood pressure measurement will be taken in your preferred exercise position. Adjustments can be made to the cycle ergometer seat height, handlebar height, and stem length to ensure you are comfortable. Next, a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip)

to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready. During the test, you will cycle against a series of increasing resistance levels (at 30W per minute). You will continue cycling on the cycle ergometer until the point your heart rate reaches 120 beats/minute. During this test, blood pressure will be taken every 3 min and your ECG trace will be monitored continuously. Following reaching the resistance level associated with a heart rate of 120 beats/minute, you will be instructed to warm down while your ECG and blood pressure are monitored. You may withdraw from the test at any time.

This session is used as a health screening session to ensure minimal risk of an adverse event during the exercise test, and to get you accustomed to wearing the oxygen mask and nose clip while exercising. This is hoped to ensure that you reach your maximal possible output during the VO_2max test.

Following Exercise

Once you have completed the familiarisation on the cycle ergometer you will be seated or directed to a bed to recover and be continually monitored. After 15 min you are able to return home following a health check to ensure you are safe to leave the premises.

The VO_2max test will be at least 12 hours after the familiarisation.

The VO_{2max} Test

Prior to the test

Once you arrive you will be taken through the Physical Activity Readiness Questionnaire again to ensure you are safe to exercise. Your body mass will be taken again, and your supine blood pressure will be verified. You will also be asked if any of your answers have changed in your heath questionnaire. Next, you will have a 12 lead ECG attached to your chest and will be seated for monitoring.

Note: At any time you may asked for the test to be stopped and have no obligation to continue with any testing.

Your VO_{2max} Test

The VO_2max test will be conducted on a cycle ergometer (exercise bike) according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by an exercise physiologist. You will then be invited to sit on the cycle ergometer and another blood pressure measurement will be recorded. When you are ready a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip) to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready. The test will involve you cycling against a series of increasing resistance levels (30W per minute) to the point at which you reach maximum oxygen output. Your blood pressure will be taken every 3 min during the test. This test will last approximately 15 min. Following reaching maximum you will be instructed to warm down while your ECG and blood pressure are monitored. You may withdraw from the test at any time. At all times during the test you must remain seated.

You may terminate the test at any time. Alternatively, we may terminate your test if:

- Adverse changes in your ECG and/or blood pressure are seen
- Any signs of poor perfusion or confusion
- Your cadence drops 15 rpm below your preferred cadence

Normal and Abnormal Responses to Maximal Exercise

During the test, it is very normal to experience increased sweatiness, heat, some shortness of breath (particularly towards the end of the test), and a local soreness in your muscles (like a burning sensation). However, it is not normal to feel tightness across your chest, light headedness, tingling in your fingertips, unusual joint pain, or unusual shortness of breath (relative to the difficulty of the exercise you are performing). If at any stage during the test you experience something that is 'unusual', please let a researcher know immediately. It is very important that you let the researcher know whether something is not right with your present health.

Following Exercise

Once you have completed the VO_2max test you will warm down on the cycle ergometer while your blood pressure and ECG are continuously monitored. Once your heart rate has dropped to below your warm up heart rate you will allowed to sit or lay on a bed to recover and be continually monitored. After 30 min you are able to return home following a health check to ensure you are safe to leave the premises.

The Repeated 1.5 hr Steady State Exercise Tests must be at least 1 day after the VO_{2max} test.

Health risks associated with a max test

With the right preparation, the VO_2max test is a safe procedure. However, even with the right preparation there is a risk that a misadventure could occur. The misadventure may include falling over, dizziness, heart attack and death. The American College of Medicine states, "exercise only provokes cardio-vascular (heart) events in individuals with pre-existing heart disease. Exercise does not provoke cardiac (heart) events in individuals with normal cardiovascular systems".

When you arrive at the Exercise Testing Laboratory at Bond University you will be asked to complete a general health questionnaire and a training questionnaire. Additionally a resting 12 Lead Electrocardiogram, resting blood pressure, blood glucose and blood lipids will be taken.

These pre-exercise-screening procedures are intended to prepare you for the VO_2max test and to ensure your safety and well-being during the exercise test.

Exercise related cardiac events in adults

The overall risk of exercise testing in a mixed subject population is approximately 4-6 cardiac events per 10,000 exercise tests. Cardiac events include heart attack, very rapid and irregular heartbeats and death.

With the correct pre-screening (preparation) before the VO_2max, and with your heartbeat, heart rhythm, and blood pressure closely monitored during the VO_2max test by an experienced exercise physiologist, the risk of cardiac misadventure is small.

Our strategies to minimize risk of a cardiac event

We will minimize the risk of a cardiac (heart) event during your VO_2max Test and Submaximal Exercise Tests by continuously monitoring you ECG and blood pressure, and by asking you to complete the following;

7. A modified physical activity readiness questionnaire (PAR-Q). The PAR-Q is a medical questionnaire which provides preliminary information about your health.
8. A medical questionnaire detailing your medical history, your family history, your smoking, drinking, and physical activity habits. You will also be asked about any drugs you may take.
9. A training questionnaire detailing your training habits of the past year.

The Exercise Trials

Following the VO_2max test you will be required to return for the two Exercise Trials (Single Exercise Bout Trial and Repeated Exercise Bout Trial). See Figure 1.

The Single Exercise Bout Trial involves a single 1.5 h submaximal exercise bout involving blood and saliva sampling before and after exercise.

The Repeated Exercise Bout Trial involves two 1.5 h submaximal exercise bouts separated by five hours involving blood and saliva sampling before and after exercise.

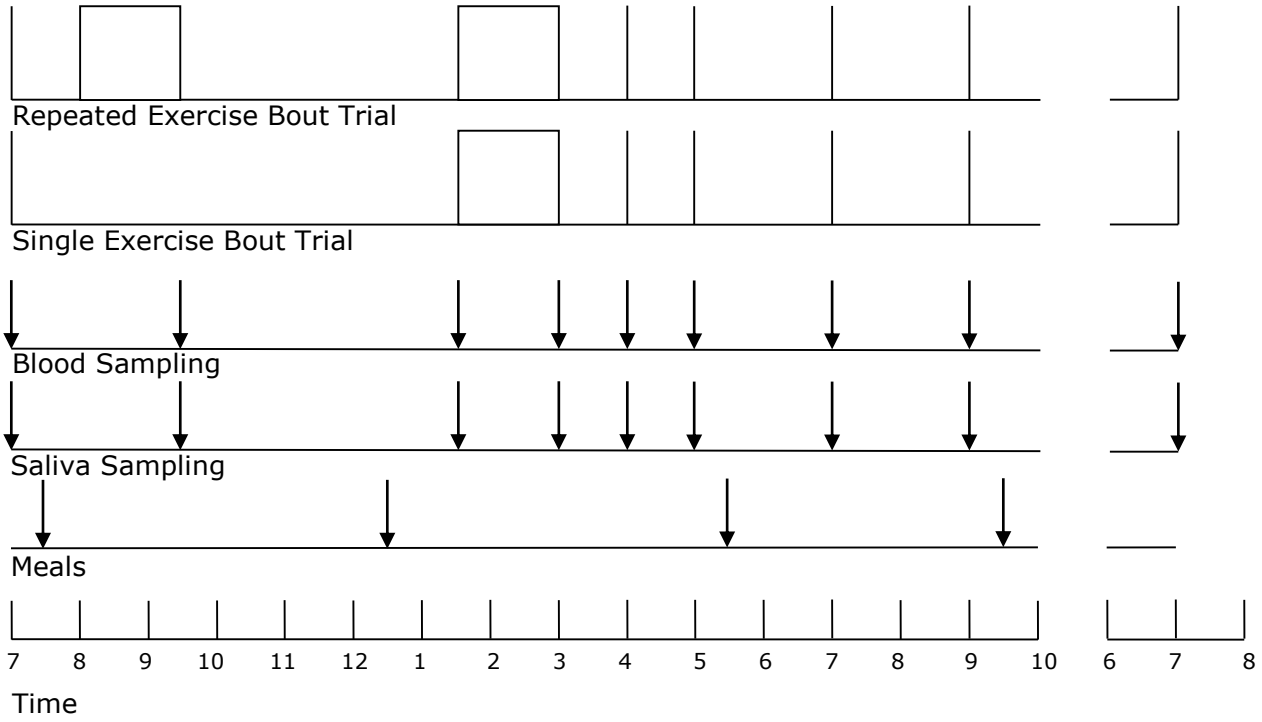

Figure 1 – Blood sampling, saliva sampling, and meal schedules during Repeated and Single Exercise Bout Trials

The Single Exercise Bout Trial and the Repeated Exercise Bout Trial will be separated by at least 3 days.

Before the day of the test

Please follow the below guidelines in the lead up to the Exercise Trials:

- Do not exercise at a high intensity within 2 days before the Exercise Trial
- Do not exercise at all the day before the before the Exercise Trial
- Do not drink alcohol the day before the Exercise Trial
- Do not drink caffeine the day of the Exercise Trial

On the day of the test

On the day of the test, please do not eat breakfast. We will take your resting blood and saliva sample and then you will be fed before you begin exercising on the bike.

Your 1.5 h Submaximal Exercise Tests

The 1.5 h Submaximal Exercise Test will be conducted on a cycle ergometer according to the American College of Sports Medicine (ACSM) Guidelines where you will be monitored by a qualified exercise physiologist. You will be taken through the PAR-Q again to ensure you are safe to exercise. Your body mass will be taken again, and your supine blood pressure will be verified. You will also be asked if any of your answers have changed in your heath questionnaire. Next, you will have a 12 lead ECG attached to your chest and will be seated for monitoring.

You will then be invited to sit on the cycle ergometer and another blood pressure measurement will be recorded. When you are ready a spirometer (oxygen mask) will be fastened to your face (with addition of a nose clip) to allow you to only breathe through your mouth into the breathing apparatus. The test will begin once you have indicated you are ready.

The test will involve you cycling against a resistance level that corresponds to 90% of your Second Ventilatory Threshold (which corresponds to approximately 75% of your VO_2max). The spirometer will not remain in your mouth the entire test, it will be used to ensure you are cycling at the correct intensity throughout; this sampling will occur every 20 min for approximately 4 min. Your blood pressure will be taken every 10 min during the test.

Following Exercise

Once you have completed each 1.5 h Submaximal Exercise Test you will be seated or directed to a bed to recover and be continually monitored. Blood and saliva samples will be collected at the time points indicated by Figure 1.

Between the sampling times of immediately post-exercise to 6 h post-exercise you will be allowed to move around within the confines of the testing area. Entertainment, including television and magazines will be provided. Meals will also be provided throughout this period.

Following the sample at 6 h post-exercise you are able to return home following a health check to ensure you are safe to leave the premises. You will not be permitted to ingest alcohol or caffeine until after the 24 h blood sample.

A call by the PhD Student/PI at approximately 1 h after leaving the premises will be made to check on you.

You are required to return for a final sample to be taken 24 h post-exercise, also you will be required to answer a health check questionnaire to ensure it is safe for you to leave the premises.

You will not be permitted to exercise between any of the blood samples (i.e. Pre-exercise to 24 hours post-exercise).

Blood Sampling

During both Exercise Trials blood samples will be taken at 9 time points (see Figure 1). The pre-exercise, immediately post-exercise and 24 h post-exercise samples will be taken by venepuncture. Samples from 1 h to 6 h post-exercise will be taken via cannulation. All blood samples will be collected by a qualified phlebotomist.

Passive Drool

A passive drool will be taken at 9 time points (see Figure 1). This 10 min passive drool will be used to test for a number of different immune variables in saliva. This can be quite an awkward process and thus it is recommended you follow these instructions to obtain the best and most efficient sample.

Prior to Collection:

- Do not eat a major meal within 60 min prior to sample collection
- Avoid alcohol consumption 24 hours prior to sample collection.

- Avoid eating dairy productions during the 30 min prior to sample collection
- Avoid acidic (e.g. Cola drinks) or high sugar foods and drinks (eg. Confectionary, sports drinks, or soft drinks) during 60 min prior to sample collection
- Do not brush your teeth within 2 hours of sample collection.
- Wash your mouth out with water 10 min prior to giving sample.

Saliva Sample Collection:

- Before you begin collection, first swallow any saliva to empty the mouth
- The collection procedure will involve a 10 min sample collection period. Passive drool spit will be collected into a 5 ml vial
- To effectively provide a sample of your salivary drool you should allow saliva to "pool" in your mouth and then without spitting, open your mouth to drool into the collection vial with your head tilted forward
- It is requested that you make minimal olfactory movements (i.e. chewing, etc.)
- This method can be repeated as often as necessary until a sufficient sample of saliva is collected within the 10 min period
- Collection tubes will be weighed before and after sample collection to determine flow rate and volume.

Benefits from you participation

You will receive a substantial amount of information about your physiological and immunological responses to exercise. We will explain the implications of all results which include; VO_2max and threshold data, markers of immune function in the respiratory tract, saliva, and blood. Furthermore, preliminarily blood glucose and blood lipid measurements will be taken to measure your risk of cardiovascular and metabolic diseases.

Information about the results of your test

Who will use the information from your results?

The results of your test will be confidential and contained within the people conducting the exercise test.

Do I get a copy of my results and feedback on participation?

You will receive your personal results and the collective results of the study of which the implication will be explained to you. This also provides an opportunity for you to ask questions.

F. Consent Form – RO955 (2009) – Study I

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Consent Form

Title: **VO_{2max} Test and 2hrs at 70% VO_{2max} Test:**

Important

The signing of this Consent Form is a very important part of your participation. The signed Consent Form is a legal document.

By signing the Consent Form, you are indicating that you clearly understand what we are asking you to do in the VO_{2max} test and 2hrs at 70% VO_{2max} test.

By signing the Consent Form, you are giving us your permission to proceed with the VO_{2max} test and 2hrs at 70% VO_{2max} test and with the procedures that we have listed in the Explanatory Statement.

By signing the Consent Form, you are indicating that you have read the Explanatory Statement, that you clearly understand what is being asked of you and that you have had all your questions about the project answered to your satisfaction.

Having signed the Consent Form, you can still withdraw from the VO_{2max} test and 2hrs at 70% VO_{2max} test at any time. You can still ask questions about any aspect of the both tests at any time.

By signing the Consent Form, you are not waiving your rights to legal action or legal representation. We can still be sued for negligence.

<u>The Formal Giving of Your Consent to participate in the:</u> VO_{2max} test and 2hrs at 70% VO_{2max} test:

Please carefully read each statement below. If you agree with each statement [(i)to(x)], would you place your initials in the box next to each statement to indicate that you understand and agree with the statement.

i. I have read the Explanatory Statement for the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (i)

ii. I understand what is being asked of me and I have had all my questions about the VO_{2max} test and 2hrs at 70% VO_{2max} test answered to my satisfaction.
Please initial in the box if you agree with the Statement (ii)

iii. I have read the section in the Explanatory Statement about the risks involved with my participation in the VO_{2max} test and 2hrs at 70% VO_{2max} test. I clearly understand the risks to my health and life by participating in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (iii)

iv. I have been told and accept the potential benefits that are expected from my participation in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (iv)

v. I understand that my results and personal information will remain confidential.
Please initial in the box if you agree with the Statement (v)

vi. I understand that I will receive feedback on my performance in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (vi)

vii. I understand that the VO_{2max} test and 2hrs at 70% VO_{2max} test will be carried out as described in the Explanatory Statement, and on that basis, I agree to participate in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (vii)

viii. I understand that you will give me a copy of the Explanatory Statement and a copy of my signed Consent Form.
Please initial in the box if you agree with the Statement (viii)

☐

ix. I understand that my participation is voluntary; that I can choose not to participate in the VO_{2max} test and 2hrs at 70% VO_{2max} test, and that I can withdraw freely at any stage from the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (ix)

☐

x. I have read the information about the taking of my blood and saliva and understand what is required of me to give a sample pre- the 2hrs at 70% VO_{2max} test immediately post, 2hrs, 4hrs, 6hrs, 8hrs, and 24hrs post 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (x)

☐

If you have initialled all boxes (i-x), would you sign in the space below indicating that you give us your consent for you to participate in the **VO_{2max} test and 2hrs at 70% VO_{2max} test.**

Name of Participant _____

Signature of Participant _____

Date _____

Witness to Signature

Name

Signature of Witness

Date

G. Consent Form – RO955 (Amended 2011) - Study II

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Consent Form

Title: **VO_{2max} Test and 2hrs at 70% VO_{2max} Test:**

Important

The signing of this Consent Form is a very important part of your participation. The signed Consent Form is a legal document.

By signing the Consent Form, you are indicating that you clearly understand what we are asking you to do in the VO_{2max} test and 2hrs at 70% VO_{2max} test.

By signing the Consent Form, you are giving us your permission to proceed with the VO_{2max} test and 2hrs at 70% VO_{2max} test and with the procedures that we have listed in the Explanatory Statement.

By signing the Consent Form, you are indicating that you have read the Explanatory Statement, that you clearly understand what is being asked of you and that you have had all your questions about the project answered to your satisfaction.

Having signed the Consent Form, you can still withdraw from the VO_{2max} test and 2hrs at 70% VO_{2max} test at any time. You can still ask questions about any aspect of the both tests at any time.

By signing the Consent Form, you are not waiving your rights to legal action or legal representation. We can still be sued for negligence.

<u>The Formal Giving of Your Consent to Participate in the:</u> VO_{2max} test and 2hrs at 70% VO_{2max} test:

Would you carefully read each statement below? If you agree with each statement [(i)to(x)], would you place your initials in the box next to each statement to indicate that you understand and agree with the statement.

xi. I have read the Explanatory Statement for the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (i)

xii. I understand what is being asked of me and I have had all my questions about the VO_{2max} test and 2hrs at 70% VO_{2max} test answered to my satisfaction.
Please initial in the box if you agree with the Statement (ii)

xiii. I have read the section in the Explanatory Statement about the risks involved with my participation in the VO_{2max} test and 2hrs at 70% VO_{2max} test. I clearly understand the risks to my health and life by participating in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (iii)

xiv. I have been told and accept the potential benefits that are expected from my participation in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (iv)

xv. I understand that my results and personal information will remain confidential.
Please initial in the box if you agree with the Statement (v)

xvi. I understand that I will receive feedback on my performance in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (vi)

xvii. I understand that the VO_{2max} test and 2hrs at 70% VO_{2max} test will be carried out as described in the Explanatory Statement, and on that basis, I agree to participate in the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (vii)

xviii. I understand that you will give me a copy of the Explanatory Statement and a copy of my signed Consent Form.
Please initial in the box if you agree with the Statement (viii)

xix. I understand that my participation is voluntary; that I can choose not to participate in the VO_{2max} test and 2hrs at 70% VO_{2max} test, and that I can withdraw freely at any stage from the VO_{2max} test and 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (ix)

xx. I have read the information about the taking of my blood and understand what is required of me to give a blood sample pre- the 2hrs at 70% VO_{2max} test immediately post, 1hr, 2hrs, 4hrs, and 6hrs post 2hrs at 70% VO_{2max} test.
Please initial in the box if you agree with the Statement (x)

If you have initialled all boxes (i-x), would you sign in the space below indicating that you give us your consent for you to participate in the **VO_{2max} test and 2hrs at 70% VO_{2max} test.**

Name of Participant

Signature of Participant

Date

Witness to Signature

Name

Signature of Witness

Date

H. Consent Form – RO1145

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Consent Form

Immune responses to repeated bouts of exercise

BUHREC Protocol Number: RO-1145

Important

The signing of this Consent Form is a very important part of your participation. The signed Consent Form is a legal document.

By signing the Consent Form, you are indicating that you clearly understand what we are asking you to do in the VO_{2max} Test and the Repeated 1.5 h Submaximal Exercise Tests.

By signing the Consent Form, you are giving us your permission to proceed with testing your VO_{2max} and immune markers during Repeated 1.5 h Submaximal Exercise Tests with the procedures that we have listed in the Explanatory Statement.

By signing the Consent Form, you are indicating that you have read the Explanatory Statement, that you clearly understand what is being asked of you and that you have had all your questions about the project answered to your satisfaction.

Having signed the Consent Form, you can still withdraw from the VO_{2max} test and Repeated 1.5 h Submaximal Exercise Tests at any time. You can still ask questions about any aspect of the both tests at any time.

By signing the Consent Form, you are not waiving your rights to legal action or legal representation. We can still be sued for negligence.

The Formal Giving of Your Consent to Participate in the: Determination of Maximum Oxygen Consumption (VO_{2max}) and measurement of immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests).

Please carefully read each statement below.

If you agree with each statement [(i) to (x)], would you place your initials in the box next to each statement, to indicate that you understand and agree with the statement.

xxi. I have read the **Explanatory Statement** for the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests)
Please initial in the box if you agree with the Statement (i)

xxii. I understand what is being asked of me and I have had **all my questions answered** about the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests)
Please initial in the box if you agree with the Statement (ii)

xxiii. I have read the section in the Explanatory Statement about the **risks involved with my participation** in the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests). I clearly understand the **risks to my health and life** by participating in these tests.
Please initial in the box if you agree with the Statement (iii)

xxiv. I have been told and accept the **potential benefits** that are expected from my participation in the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests).
Please initial in the box if you agree with the Statement (iv)

xxv. I understand that my results and personal information will **remain confidential**.
Please initial in the box if you agree with the Statement (v)

xxvi. I understand that I will receive **feedback** on my performance in the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests).
Please initial in the box if you agree with the Statement (vi)

xxvii. I understand that the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests) will be **carried out as described** in the Explanatory Statement, and on that basis, I agree to participate in the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests).
Please initial in the box if you agree with the Statement (vii)

xxviii. I understand that you will give me a copy of the **Explanatory Statement** and a copy of my signed **Consent Form**.
Please initial in the box if you agree with the Statement (viii)

xxix. I understand that my participation is **voluntary**; that I can choose not to participate in the VO_{2max} test and immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests), and that I can **withdraw freely at any stage** from these tests.
Please initial in the box if you agree with the Statement (ix)

xxx. I have read the information about the **taking of my blood and saliva** and understand what is required of me to give a blood and saliva sample before the familiarisation session, before each 1.5 h Submaximal Exercise Tests, immediately post-, 1 h, 2 h, 4 h, 6 and 24 h post-exercise.
Please initial in the box if you agree with the Statement (x)

If you have initialled all boxes (i-x), would you sign in the space below indicating that you **give us your consent for you to participate** in the VO_{2max} Test and measurement of immune markers during repeat bouts of exercise (Repeated 1.5 h Submaximal Exercise Tests).

Name of Participant

Signature of Participant

Date

I. Medical History Questionnaire

RISK STRATIFICATION MATRIX

POSITIVE RISK FACTORS	DEFINING CRITERIA
Age	Men ≥45 yr; Women ≥55 yr
Family History	Myocardial infarction, coronary revascularisation, or sudden death before 55 yr of age in father or other male first-degree relative, or before 65 yr of age in mother or other female first degree relative
Cigarette smoking	Current cigarette smoker or those who quit within the previous 6 months or exposure to environmental tobacco smoke
Sedentary lifestyle	Not participating in at least 30 min of moderate intensity (40%−60% VO2max) physical activity on at least three days of the week for at least three months
Obesity[a]	Body mass index ≥30 kg.m2 or waist girth >102 cm for men and >88 cm for women
Hypertension	Systolic blood pressure ≥140 mmHg and/or diastolic ≥90 mm Hg, confirmed by measurements on at least two separate occasions, or on antihypertensive medication
Dyslipidemia	Low-density lipoprotein (LDL-C) cholesterol ≥130 mg.dL-1 (3.37 mmol.L-1) or high-density lipoprotein (HDL-C) cholesterol <40 mg.dL-1 (1.04 mmol.L-1) or on lipid-lowering medication. If total serum cholesterol is all that is available use ≥200 mg.dL-1 (5.18 mmol.L-1)
Prediabetes	Impaired fasting glucose (IFG) = fasting plasma glucose ≥100 mg.dL-1 (5.5 mmol.L-1) but <126 mg.dL-1 (6.93 mmol.L-1) or impaired glucose tolerance (IGT) = 2-hour values in oral glucose tolerance test (OGTT) ≥140 mg.dL-1 (7.70 mmol.L-1) but <200 mg.dL-1 (11.00 mmol.L-1)

NEGATIVE RISK FACTORS	DEFINING CRITERIA
High-serum HDL cholesterol	≥60 mg.dL-1 (1.55 mmol.L-1)

Note: It is common to sum risk factors in making clinical judgements. If HDL is high, subtract one risk factor from the sum of positive risk factors, because high HDL decreases CVD risk.

[a] Professional opinions vary regarding the most appropriate markers and thresholds for obesity; therefore, allied health professionals should use clinical judgement when evaluating this risk factor.

KNOWN CARDIOVASCULAR, PULMONARY, OR METABOLIC DISEASE

Cardiovascular	Cardiac, peripheral vascular, or cerebrovascular disease
Pulmonary	COPD, asthma, interstitial lung disease, or cystic fibrosis
Metabolic	Diabetes mellitus, (types 1 and 2), thyroid disorders, renal or liver disease

MAJOR SIGNS OR SYMPTOMS SUGGESTIVE OF CARDIOVASCULAR, PULMONARY, OR METABOLIC DISEASE

- Pain, discomfort in the chest, neck, jaw, arms, or other areas that may result from ischemia
- Shortness of breath at rest or with mild exertion
- Dizziness or syncope
- Orthopnea or paroxysmal nocturnal dyspnea
- Ankle oedema
- Palpitations or tachycardia
- Intermittent claudications
- Known heart murmur
- Unusual fatigue or shortness of breath with usual activities

RISK STRATIFICATION CATEGORIES

1.	Low risk threshold	Individuals who are asymptomatic and meet no more than one risk factor
2.	Moderate risk factors	Those who are asymptomatic who meet the threshold for two or more risk
3.	High risk	Individuals with one or more signs or symptoms suggestive of, or individuals with known cardiovascular, pulmonary, or metabolic disease

J. Exercise History Questionnaire

Bond University
Faculty of Health Sciences & Medicine

Exercise Immunology Study

Training Questionnaire

Criteria to be used

Subject Inclusion criteria:

1. Males aged 18-35 years
2. A minimum of 1 year cycling racing experience.
   Currently racing in Elite A and/or A Grade club racing.
3. Health Insurance (i.e. Australian residence and/or citizens).

Subject Exclusion Criteria:

1. Contraindications or risk to exercise (recent injuries and/or overt contraindicators for performing maximal exercise as defined by risk stratification matrix).
2. Thyroid and/or other autoimmune disease
3. Primary or secondary immune deficiency

If you are unsure about any of the following questions please seek clarification before responding.

Subjects name:

1. On average over the past 3 months, approximately how many kilometres per week have you trained?

2. On average how many sessions per week do you train?

3. Do you compete in any other sport competitively? YES NO

   Sport:

   If so, what is you training/competition regime?

4. Approximately how often do you compete in cycling races (For example, once a week/once a month/etc.)? Please include the type of racing (e.g. criteriums/road races/time trials/triathlon/mountain biking)

5. How many years have you been competing in cycle racing?

6. Please specify your current racing Grades?

   Club level:

   Open Grading:

7. Are you currently suffering from any chronic (long-term) injury that you feel may affect your ability to reach maximum outputs on a bike? Give details if relevant:

8. Do you use any nutritional supplements? YES NO

   Please specify (such as vitamin C, multivitamin, protein powder, etc.)

9. Do you have a thyroid disorder, or any other autoimmune disorder (such as Type I Diabetes/Addison's Disease)?

   YES NO

10. Do you a primary (such as selective IgA deficiency) or secondary (such as Hepatitis, HIV) immune deficiencies?

    YES NO

11. Family History – Indicate if any of your immediate family (grandparents, parents, brothers, sisters) have experienced any of the following (list their age and relationship to you):

	Relationship and Age
Heart Disease	
Stroke	
Diabetes	
Cancer	

12. Do you have Health Insurance? YES NO

Emergency Contact Form

In case of emergency I authorise the research team, or a representative thereof, to contact the following persons.

Person One

Name:

Relationship:

Phone Number (s):

Person Two

Name:

Relationship:

Phone Number (s):

I have provided a truthful response to all of these questions.

Signed: Date:

K. Physical Activity Readiness Questionnaire

L. Individual Subject Characteristics – Study I

Study I Subject	Age	Height	Body Mass	BMI	FVC	FEV_1	FEV_1/FVC	Supine HR	Supine Systolic BP	Supine Diastolic BP	Training	Absolute VO_2peak	Relative VO_2peak
1	23	1.86	73.50	21.15	6.46	5.18	80.12	42	130	74	250	4.61	62.7
2	18	1.87	74.20	21.26	6.26	4.95	79.02	48	120	80	450	4.41	59.4
3	22	1.82	71.10	21.54	5.92	4.85	82.00	50	124	80	275	5.35	75.3
4	26	1.93	88.70	23.86	6.43	5.21	81.02	45	110	66	350	4.97	56
5	19	1.78	67.10	21.08	5.54	4.67	84.23	53	110	70	300	4.76	70.9
6	34	1.75	64.50	21.18	5.46	4.27	78.12	41	110	68	400	4.87	75.5
7	24	1.80	72.80	22.57	5.69	4.91	86.21	45	120	80	300	5.26	72.3
8	20	1.80	74.50	22.89	5.74	4.61	80.30	57	118	74	150	4.37	58.7
9	24	1.72	73.90	24.86	5.45	4.36	79.93	43	128	68	250	4.79	64.8
10	32	1.92	77.60	21.16	6.84	5.56	81.29	36	122	76	200	4.92	63.5
Mean	24.20	1.82	73.79	22.16	5.98	4.85	81.22	46.00	119.20	73.60	292.50	4.83	65.91
SD	5.27	0.07	6.47	1.34	0.49	0.40	2.42	6.16	7.32	5.40	89.79	0.32	7.12

M. Individual Subject Characteristics – Study II

Subject	Age	Height	Body mass	BMI	Fasting Glucose	HDL	LDL	Total Chol.	HDL/ Chol.	FVC	FEV_1	FEV_1/ FVC	Supine HR	Supine Systolic	Supine Diastolic
1	31	1.79	70.9	22.13	4.4	1.72	2.28	4.03	0.43	6.06	4.87	80.36	42	130	74
2	22	1.79	67.5	21.07	4.8	0.94	2.55	3.42	0.27	5.43	4.39	80.85	48	120	80
3	30	1.79	80.8	25.22	4.5	1.04	4.19	4.68	0.22	6.5	5.27	81.08	50	124	80
4	20	1.77	76	24.26	5.4	1.16	4.03	3.13	0.37	5.72	4.97	86.89	45	110	66
5	24	1.81	70.7	21.58	4.6	1.7	3.1	5	0.34	5.76	4.99	86.63	53	110	70
6	26	1.93	81.6	21.91	4.7	1.24	2.27	2.87	0.43	6.6	5.28	80.00	41	110	68
7	18	1.81	72.2	22.04	3.3	0.84	2.9	2.65	0.32	5.3	4.42	83.40	45	120	80
8	23	1.79	65.3	20.38	3.8	1.19	1.69	3.37	0.35	6.24	5.12	82.05	57	118	74
9	24	1.87	79.3	22.68	4.2	1.29	4.16	5.74	0.22	7.36	5.03	68.34	43	128	68
10	22	1.76	68.4	22.08	4.3	1.5	1.87	2.77	0.54	5.8	4.7	81.03	36	122	76
Mean	24.00	1.81	73.27	22.33	4.40	1.26	2.90	3.77	0.35	6.08	4.90	81.06	46.00	119.20	73.60
SD	4.08	0.05	5.81	1.43	0.57	0.30	0.94	1.06	0.10	0.62	0.31	5.11	6.16	7.32	5.40

Subject	Training	Absolute VO2peak	Relative VO2peak	Peak Power	Peak W/kg	VT1 Power	VT1 W/kg	VT2 Power	VT2 W/kg	Mean HR	Mean VO2	Mean Cadence	Mean Power
1	480	4.71	66.43	505	7.12	300	4.23	345	4.87	141.07	3.375	100.2	315
2	400	4.46	66.07	405	6.00	278	4.11	330	4.89	163.56	3.537	98.7	280
3	350	4.74	58.66	465	5.75	330	4.08	353	4.36	156.84	3.582	95.9	300
4	300	4.74	62.37	435	5.72	270	3.55	338	4.44	140.43	3.5415	92.8	285
5	300	4.66	65.91	450	6.36	300	4.24	383	5.41	174.95	3.6225	90.9	315
6	300	5.03	61.64	522	6.40	330	4.04	405	4.96	142.15	3.9645	91.6	330
7	350	4.17	57.76	420	5.82	263	3.64	323	4.47	157.97	3.1635	96.2	285
8	350	3.90	59.72	405	6.20	270	4.13	330	5.05	150.18	3.123	95.6	285
9	350	4.18	52.71	435	5.49	285	3.59	338	4.26	158.22	3.1545	94	290
10	300	3.82	55.85	410	5.99	245	3.58	320	4.68	152.18	2.907	87.5	275
Mean	348.00	4.44	60.71	445.20	6.09	287.00	3.92	346.25	4.74	153.76	3.40	94.34	296.00
SD	57.12	0.40	4.64	41.10	0.47	28.03	0.29	27.37	0.36	10.96	0.31	3.79	18.23